Competitive Binding of Amino-acids and Nucleic Acid Constituents towards the Nitrosyliron Paramagnetic Probe

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The competitive binding of amino-acids and nucleic acid constituents with the $Fe^{I}(NO)_2$ group has been studied. The Fermi contact interaction between the metal ion and the ligand nuclei has been investigated by means of isotopic substitution and computer simulation. Comparable binding powers have been shown by amino-acid and nucleic acids site, depending on pH. The availability of an ionized mercapto group in amino-acid side chains strongly modifies the co-ordination behaviour.

SPECIFIC interactions between amino-acid side chains and nucleic acid constituents allow the selective recognition of nucleic acid base sequences by proteins. These interactions may be direct or indirect. Stacking, hydrogen bonding, and electrostatic binding result from direct interactions,[1-5] flexibility and specificity of biological reactions are due to such non-covalent interactions.[6]

Furthermore the specific interaction between aromatic groups of proteins and nucleic acids may be also favoured by the presence of metal [7] ions which act as ionic bridges. In this way metal bridged adducts of increased stability are formed even if the role of metal ions in protein–nucleic acid complexes has not yet been elucidated in

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most cases.[8-16] For example, it is not well known how a ligand bound to a metal ion affects the metal-binding of another ligand: this is the key of most catalytic biological processes. A great deal of work has been devoted to elucidate this general problem, by means of n.m.r.,[17-19] *X*-ray diffraction,[20] fluorescence, and c.d.[21,22] techniques.

However N(7) in the purine ring and the phosphate group are certainly involved and play the most important role in these reactions.[23-28] It is useful to investigate the co-ordination behaviour of these sites and the competitive binding to a paramagnetic probe in relation to amino-acids at the physiological pH value. From the e.s.r. spectra of the $Fe^{I}(NO)_2$ complexes in solution, it is possible to draw conclusions on the binding strength of the various groups involved in metal co-ordination and on the simultaneous presence of different metal complexes.[29-31]

In spite of the fact the $Fe^{I}(NO)_2$ group is known to be little involved in biological systems [29,32] it is a valuable paramagnetic probe as it displays a well resolved hyperfine structure. The presence of the two groups stabilizes the +1 oxidation state of the iron atom, which may be significant in a biological context.

The aim of this paper is to compare the binding strength of various amino-acids relative to nucleic acid bases with the nitrosyliron probe.

EXPERIMENTAL

A Varian V-4502 100 kHz field modulation e.s.r. spectrometer, operating in the microwave X-band, was used in performing the e.s.r. experiments. Fremy's salt (g 2.005 5; a_N 1.30 mT) was used as an external reference standard for the calibration of the e.s.r. parameters. The microwave frequency was measured by a Hewlett–Packard model X5-32B frequency meter. Nuclear hyperfine constants (a) were measured by comparison with those of computer-simulated spectra. These were taken by assuming a purely Lorentzian line shape. The variables were the coupling constants, the relative intensities of the hyperfine lines, and the electron spin relaxation times.

Water solutions of $Fe(ClO_4)_2,6H_2O$ (Alpha Inorganics) (5×10^{-4}—5×10^{-2}M) were prepared and saturated with gaseous NO, obtained from $NaNO_2$ by previously reported methods.[28,33] Proper amounts of ligands were added to the nitrosyliron solutions. The pH was varied with NaOH or $HClO_4$ and measured by means of a Metrohm–Herisau E-516 Titriskop pH meter. Sodium nitrite (^{15}N 99.9%) was obtained from ICN Chemicals; other chemical and biological compounds were obtained from Merck and Fluka. They were reagent grade and used without further purification.

TABLE 1

E.s.r. data for complexes formed from amino-acids and $Fe(NO)_2$(adenine)

$Fe(NO)_2$(adenine) derivative	pH	g Value	Number of lines (^{14}NO)	$a_{^{14}NO}$/mT	Number of lines (^{15}NO)	$a_{^{15}NO}$/mT	a_N/mT	a_H/mT
Adenine	8	2.027	9	0.20	7	0.28	0.27	
Imidazole	9	2.027	9	0.20	7	0.28	0.27	
α-Alanine	7	2.033 + 2.019	1 + 9	0.33	5	0.46	0.66	
Φ-Alanine	7	2.033 + 2.019	1 + 9	0.33	5	0.46	0.66	
Serine	7	2.033 + 2.015	1 + 9	0.33	5	0.46	0.66	
Tryptophan	7	2 027 + 2.019	9 [a]	0.20	7 + 5	0.28 + 0.46	0.27 + 0.66	
N-Acetylcysteine	9—10	2.029	13	0.24	9	0.33		0.14
	6—7	2.029	3 + 13	1.32 + 0.24	2 + 9	1.85 + 0.33		0.14
Cysteine methyl ester	9—10	2.029	13	0.24	9	0.33		0.14
	6—7	2.031	11	0.21			0.44	0.21
S-Methylcysteine	8—9	2.019	9	0.33	5	0.46	0.66	
	6—7	2.027	9	0.20	7	0.28	0.27	
Histidine methyl ester	8—9	2.033	9	0.21	7	0.28	0.27	

[a] With hyperfine splitting.

RESULTS AND DISCUSSION

Competitive Binding between Amino-acids and $Fe^{I}(NO)_2$(*adenine*) *Complex.* It has been shown [30] that the e.s.r.

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spectrum of adenine with the $Fe^{I}(NO)_2$ group displays a nine-line pattern at g 2.027—2.028 which converts to a septet in the presence of ^{15}NO (relative intensities 1 : 4 : 8 : 10 : 8 : 4 : 1). These findings were interpreted in terms of a $Fe^{I}(NO)_2$(adenine)$_2$ complex, where N(7) in the imidazole ring is the preferred binding site in the pH range 7—9.5.

The interaction of the $Fe(NO)_2$(adenine) complex with

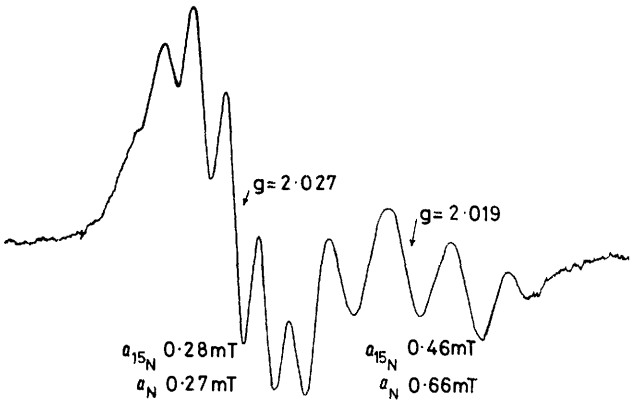


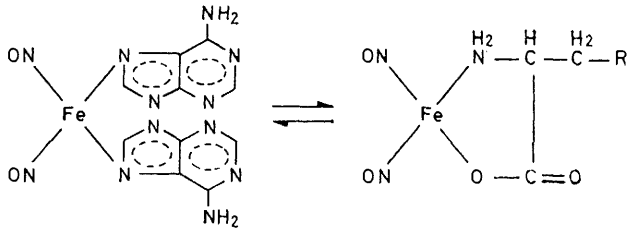
FIGURE 1 Experimental e.s.r. spectrum of the $Fe(^{15}NO)_2$–adenine–trptophan system at pH 7

various amino-acids in solution was studied in order to obtain information about the competitive co-ordination behaviour of the ligands. Table 1 summarizes the e.s.r. parameters of the starting complex compared with those formed in solution after ligand addition. α-Alanine at neutral pH gives rise to a drastic modification of the e.s.r. spectrum which display the characteristic $Fe(NO)_2$(amino-acid) e.s.r. pattern [29] at g 2.019. Similar results are obtained from phenylalanine and serine, where metal chelation *via* the carboxy and amino groups is by far favoured over metal binding to N(7) in the imidazole ring. A different situation is shown by tryptophan, whose e.s.r. spectra strongly point to a pH dependent equilibrium between different types of $Fe(NO)_2$ complexes.

Figure 1 shows the results after isotopic substitution with ^{15}NO. In this case slow exchange conditions hold, leading to large overlap of the e.s.r. patterns due to the adenine and tryptophan complexes.

The dynamic aspects of the metal–ligand complexes formation may be related to competitive co-ordination behaviour of different binding sites. The existence of both complexes in equilibrium has been proved by the variation of the relative intensity with pH, showing comparable linkage capability at physiological pH values.

Some ' protected ' amino-acids containing the mercapto group, whose important role is well known,[28-31] were then considered. By adding small amounts of *N*-acetylcysteine to $Fe^{I}(NO)_2$(adenine)$_2$ solution, an e.s.r. signal at g 2.029 was obtained. The 13 hyperfine lines (relative intensities 1 : 4 : 8 : 12 : 16 : 20 : 22 : . . .) arise from unpaired electron interaction with two ^{14}NO nitrogen nuclei and four protons (in methylene groups) adjacent to sulphur atoms which are directly bound to



iron. An equilibrium of a mononitrosyl complex with the amino-acid has been proposed [31,34] on the basis of the simultaneous presence of a triplet at lower field with a_{NO} 1.32 mT. This interpretation has been verified and the coupling constant values corrected by means of isotopic substitution (nine lines) and computer simulation techniques. The new values are $a_{^{15}NO}$ 0.24, a_{CH_2} 0.14, $a_{^{15}NO}$ 0.33, ΔH 0.09 mT. Hindrance at the NH_2 site promotes mercapto-group involvement in the binding provided the pH allows its ionization.

Similar behaviour has been shown in the pH range 9—10 by cysteine methyl ester whereas a drastic difference is apparent at neutral pH. In fact nuclear

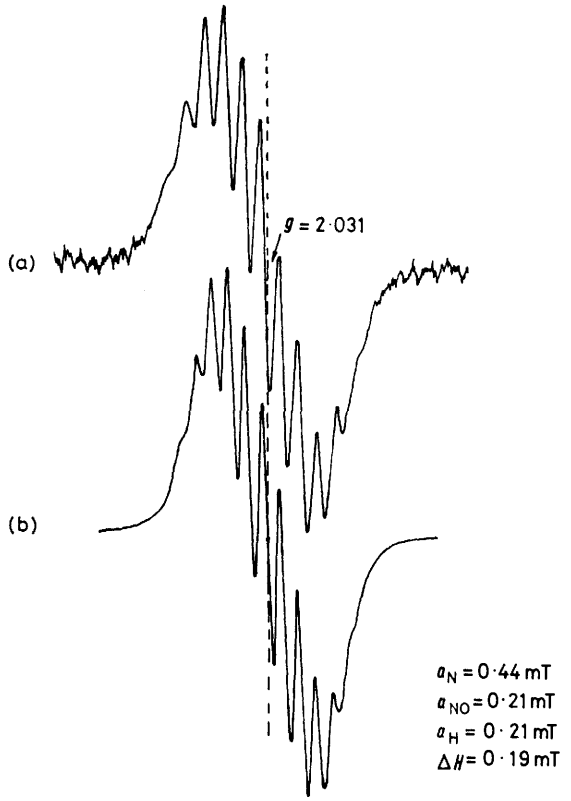
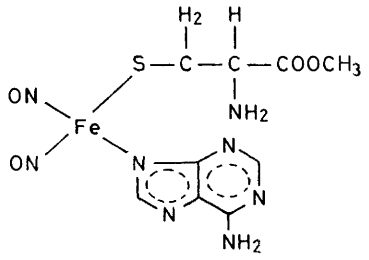


FIGURE 2 (*a*) Experimental e.s.r. spectrum of the $Fe(^{14}NO)_2$–adenine–cysteine methyl ester ternary complex at pH 7; (*b*) simulated e.s.r. spectrum

hyperfine splitting at g 2.031 with relative intensities 1 : 4 : 9 : 14 : 17 : 18 : 17 : 14 : 9 : 4 : 1 indicates the

[34] C. C. McDonald, W. D. Phyllips, and F. Mower, *J. Amer. Chem. Soc.*, 1965, **87**, 3319.

formation of a ternary complex in solution containing the paramagnetic probe, adenine, and the amino-acid, which binds *via* NH_2. Computer simulation (Figure 2) establishes the existence of this mixed complex and



(1)

indicates that a mixture of the corresponding binary parent complexes is not present. It is possible to suggest structure (1) for the complex. Such complex formation at physiological pH values suggests biological implications where mixed co-ordination is of prime importance.

In order to investigate the relevance of the mercapto group to the metal binding behaviour, *S*-methylcysteine was examined. In this case, the amino-acid ligand ability *via* CO_2^- and NH_2 is shown only at basic pH, while at neutral pH the adenine complex is by far the most stable.

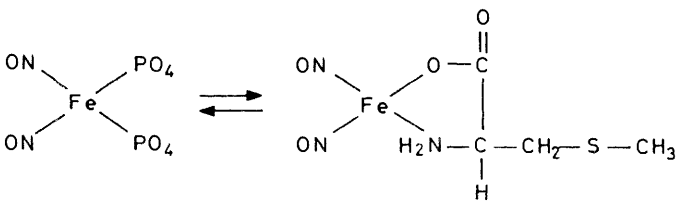
It is noteworthy that a study of $Fe^I(NO)_2$(imidazole)$_2$ competitive binding relative to the previously described amino-acids gives rise to identical features indicating the relevance of the imidazole site in the co-ordination behaviour of adenine.

Competitive Binding between Amino-acids and $Fe^I(NO)_2$-(*Phosphate*) *Complex*.—A reinvestigation, by means of computer simulation, of the e.s.r.[34] of dinitrosyl(phosphate)iron complexes, provides more accurate values for nuclear hyperfine constants. Table 2 shows these new values compared with those obtained after addition of amino-acids. It can be seen that α-alanine and *S*-methylcysteine give rise to an almost identical spectrum at neutral pH. The similar behaviour of these amino-acids is explained in terms of previously reported considerations.

Figure 3 shows experimental and simulated e.s.r. spectra, which were obtained after addition of *S*-methylcysteine to a $Fe^I(NO)_2$(phosphate)$_2$ solution. The septet at *g* 2.033 is clearly attributable to an $Fe^I(NO)_2$-(phosphate)$_2$ complex in which the number and the relative intensities of the hyperfine components are consistent with a contact interaction involving two equivalent ^{14}N nuclei (by ^{14}NO) and two ^{31}P nuclei (*I* 1/2) with splitting constants $a_{^{14}N}$ 0.24 and $a_{^{31}P}$ 0.30 mT, respectively. The 9-line spectrum at *g* 2.019, which partially overlaps the septet, is due to the characteristic amino-acid pattern.

Figure 4 shows the modifications after selective ^{15}NO isotopic substitution in the experimental and simulated spectra. Despite the partial overlapping of the e.s.r. sequences, the spectra provide an unambiguous interpretation.

These results are explained in terms of the pH dependent equilibrium (*1*). It is noteworthy that at neutral



(1)

pH the binding strength of the phosphate group and the amino-acids is very similar. In contrast with these findings, cysteine methyl ester gives rise only to the previously discussed 13-line pattern at *g* 2.029 showing that the co-ordination *via* S^- is stronger than *via* phos-

TABLE 2

E.s.r. data for complexes from amino-acids and $Fe(NO)_2$(phosphate)

$Fe(NO)_2$(phosphate) derivative	pH	*g* Value	Number of lines (^{14}NO)	$a_{^{14}NO}$/mT	Number of lines (^{15}NO)	$a_{^{15}NO}$/mT	a_N	a_H	a_P
Phosphate	7	2.033	7	0.24	5	0.34			0.30
α-Alanine	7	2.033 + 2.019	7 [a]	0.24	5 + 5	0.34 + 0.46	0.66		0.30
Cysteine methyl ester	7	2.029	13	0.24	9	0.33		0.14	
S-Methylcysteine	7	2.033 + 2.019	7 + 9	0.24 + 0.33	5 + 5	0.34 + 0.46	0.66		0.30

[a] With hyperfine splitting.

TABLE 3

E.s.r. data for complexes of amino-acids and $Fe(NO)_2$(2-thiouracil)

$Fe(NO)_2$(2-thiouracil)	pH	*g* Value	Number of lines (^{14}NO)	$a_{^{14}NO}$/mT	Number of lines (^{15}NO)	$a_{^{15}NO}$/mT	a_N/mT	a_H/mT
2-Thiouracil	4—6	2.021	9	0.24	5	0.34	0.40	
	6—8	2.029	5	0.23	3	0.32		
α-Alanine	5.5—7	2.029 + 2.021	5 + 9	0.23 + 0.24	3 + 5	0.32 + 0.34	0.40	
N-Acetylcysteine	9	2.029	13	0.24	9	0.33		0.14
Cysteine methyl ester	7—9	2.029	13	0.24	9	0.33		0.14
S-Methylcysteine	7—8	2.029 + 2.021	5 + 9	0.23 + 0.24	3 + 5	0.32 + 0.34	0.40	

phate. The e.s.r. results for *S*-methylcysteine (Table 2) confirm this.

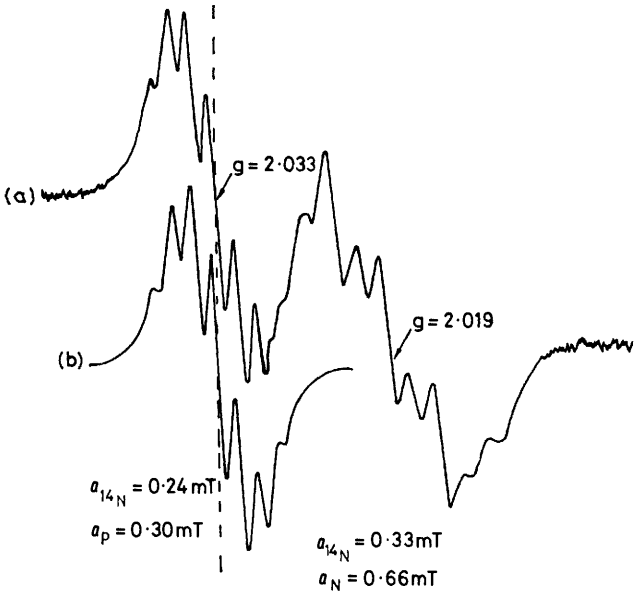


FIGURE 3 (a) Experimental e.s.r. spectrum of the $Fe(^{14}NO)_2$–phosphate–*S*-methylcysteine system at pH 7; (b) simulated e.s.r. spectrum of the $Fe(^{14}NO)_2(phosphate)_2$ complex

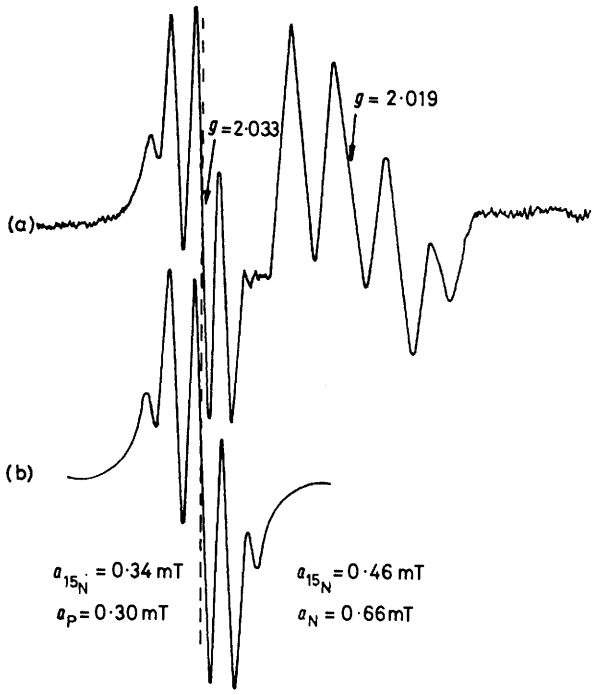


FIGURE 4 (a) Experimental e.s.r. spectrum of the $Fe(^{15}NO)_2$–phosphate–*S*-methylcysteine system at pH 7; (b) simulated e.s.r. spectrum of the $Fe(^{15}NO)_2(phosphate)_2$ complex

Competitive Binding between Amino-acids and $Fe^{I}(NO)_2$*(2-thiouracil) Complex.*—The importance of the mercapto group both in amino-acids and thio-bases must be closely examined. The nitrosyliron complex with 2-thiouracil,[31] which displays anti-tumor and anti-thyroid activity, has been studied. Table 3 shows the e.s.r. parameters compared with those obtained after addition of some thio- and non-thio-substituted amino-acids. Whenever the thio group of the amino-acid has ligand activity (*i.e.* in *N*-acetylcysteine and cysteine methyl ester), it is by far the preferred binding site. On the contrary, whenever S^- is not present, (*i.e.* in α-alanine and *S*-methylcysteine), the thio-base is preferentially involved in metal co-ordination. In spite of the fact that α-amino-acids and thiopyrimidines show similar e.s.r. patterns at *g ca.* 2.020,[29,31] both with ^{14}NO and ^{15}NO, analysis of the coupling constants provides an unambiguous interpretation (Table 3). Figure 5 displays the e.s.r. spectrum of the $Fe^{I}(NO)_2$(2-thiouracil)$_2$ system in the presence of α-alanine.

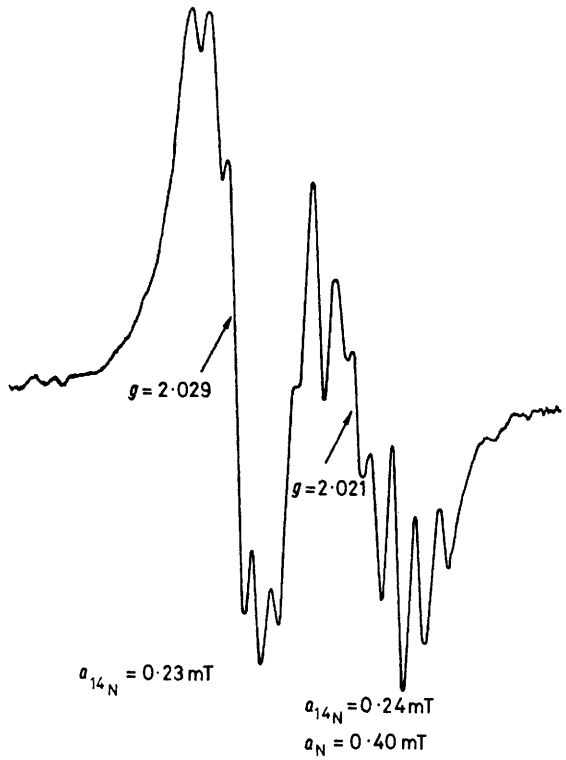


FIGURE 5 Experimental e.s.r. spectrum due to the $Fe^{I}(NO)_2$-(2-thiouracil)$_2$ system in the presence of α-alanine at neutral pH

Conclusions.—For biologically significant pH values, the binding sites of nucleic acid bases compete with those of amino-acids for metal co-ordination. The simultaneous binding of metal ion to N(7) of adenine and to NH_2 of cysteine methyl ester is worth noting. The implications of pH variation in these competitive equilibria are obvious. Whenever there is a deprotonated thio group in the amino-acid side chain co-ordination to the metal probe is *via* S^- in preference to the remaining binding sites.

Though caution should be exercised in applying conclusions drawn from model systems to enzyme reactions, the results for formation of mixed ligand complexes provide a basis for meaningful design and interpretation of experiments in biochemical catalysis.

In particular, the investigation of the interaction between iron and cysteine derivatives may provide some insight into the nature of the active sites of ferredoxins. Several studies on iron–sulphur co-ordination compounds [35,36] allowed some suggestions concerning the structural and redox properties of these proteins to be made. Interesting correlations between g values, covalency, and redox potential have been made.[37] The e.s.r. investigation of mononitrosyl iron–sulphur complexes, rather than the dinitrosyl compounds, may give further information on the molecular structure of the metal–ligand cluster in ferredoxins.[38,39] From this point of view, there is only one relevant case in our study, the [Fe(NO)(N-acetylcysteine)$_2$] complex at neutral pH with g 2.027 and a_N 1.32 mT.

We are grateful to Professor E. Perroni and to C.N.R. for facilities offered at the Institute of Physical Chemistry, Florence, Italy, and for the use of the e.s.r. spectrometer.

[7/1453 *Received*, 9*th August*, 1977]

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