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Amino Terminal Cu(II)- and Ni(II)-Binding (ATCUN) Motif of Proteins and Peptides: Metal Binding, DNA Cleavage, and Other Properties†

CATHERINE HARFORD‡ AND BIBUDHENDRA SARKAR*

Department of Biochemistry Research, The Hospital for Sick Children, Toronto, Ontario, M5G 1X8, Canada, and Department of Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada

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Introduction

The amino terminal Cu(II)- and Ni(II)-binding (ATCUN) motif has been studied for more than 35 years.[1] During that time, our range of knowledge of the motif has expanded from a small metal-binding site on albumins to a Cu(II)- and Ni(II)-binding motif which can be found in other naturally occurring proteins and which can be designed into proteins to provide them with DNA cleavage capability. The ATCUN motif is a structural feature which can be defined as being present in a protein or peptide which has (1) a free NH_2-terminus, (2) a histidine residue in the third position, and (3) two intervening peptide nitrogens. The ATCUN motif occurs naturally in certain species of albumins (e.g., HSA, BSA, and RSA), neuromedins C and K, human sperm protamine P2a, and histatins. The ATCUN motif binds Cu(II) and Ni(II) specifically, but can release the metal easily with appropriate ligands, reflecting its role as a transport site in albumins. In this Account, we will discuss the origin of the ATCUN motif from the early characterization of the metal-binding properties within albumins, molecular design of the ATCUN motif, DNA cleavage properties of ATCUN motif, use of the motif in protein design for the specific DNA cleavage, and prediction of naturally occurring proteins with the ATCUN motif.

Catherine Harford was born August 24, 1963, in Toronto, Canada. She started her research as an undergraduate summer student in Dr. B. Sarkar's laboratory, later was admitted to medical school while continuing as a summer student in his laboratory, and finally returned to complete her B.Sc. (Honors) (1989) and Ph.D. (1995) in biochemistry from the University of Toronto under his supervision. Currently she is doing postdoctoral research at The Massachusetts General Hospital, Harvard Medical School, Boston.

Bibudhendra Sarkar was born August 2, 1935, in Kushtia (then India, later E. Pakistan, now Bangladesh). He received his B.Pharm. (1956) and M.Pharm. (1957) from Banares University, India. He came to the United States in 1959 and received his Ph.D. (1964) in biochemistry from the University of Southern California, Los Angeles. In the same year, he joined the Research Institute of the Hospital for Sick Children, Toronto, Canada, where he is now the Head of the Department of Biochemistry and Professor of Biochemistry, University of Toronto. He has been a Visiting Professor and Scholar in the Universities of Paris (France) and Cambridge (U.K.). He developed the copper−histidine therapy for Menkes disease, a fatal neurodegenerative disease in children caused by a genetic defect of copper transport. He has received several awards and honors including MRC Scholar (Canada) and Nuffield Foundation (U.K.) Awards and was an invited speaker at the Nobel Symposium on Inorganic Biochemistry in Sweden. He is a Fellow of the Chemical Institute of Canada and a member of ACS, AAAS, ASBMB, CSBMCB, Protein Society, etc. His research focuses on inorganic biochemistry with major interests in the therapies of Menkes and Wilson diseases, protein design involving metals, and genotoxic effects of metal substitution in zinc finger proteins.

Albumins: Origin of the ATCUN Motif

The ATCUN motif was first characterized in albumin. Albumin (MW 69 000) is the most abundant protein in plasma (4.5 g/100 mL).[2] One of its most prominent functions is transport of several small molecules and ions including metals.[3] This laboratory has characterized albumin's metal-binding sites extensively. Metals studied have included Cu(II), Ni(II), Cd(II), and Zn(II).[4−22] The most well characterized metal−albumin interactions involve Cu(II) and Ni(II).

Copper(II)−albumin is the major transport form of Cu(II) in blood. However, only 5−10% of serum copper (total serum concentration 108 μg/dL) is bound to albumin; most of the rest is bound to ceruloplasmin, with the remainder being bound to peptides and amino acids such as histidine.[23] In contrast, 95% of serum Ni(II) (total serum concentration 0.6 μg/dL) is bound to albumin, and the rest is bound to histidine.[2,24] Copper(II) can be bound to albumin as a Cu(II)−albumin complex or as a ternary complex with amino acids such as histidine.[4] Under physiological conditions, there is an equilibrium among

* To whom correspondence should be addressed at the Department of Biochemistry Research, The Hospital for Sick Children, 555 University Ave., Toronto, Ontario M5G 1X8, Canada. Phone: (416) 813-5921. Fax: (416) 813-5379.

† Abbreviations used: ATCUN motif, amino terminal Cu(II)- and Ni(II)-binding motif; BSA, bovine serum albumin; bZIP, basic leucine zipper; CSA, chicken serum albumin; DSA, dog serum albumin; ESR, electron spin resonance; HSA, human serum albumin; NMR, nuclear magnetic resonance; PSA, porcine serum albumin; RSA, rat serum albumin.

‡ Current address: Department of Surgery, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114.

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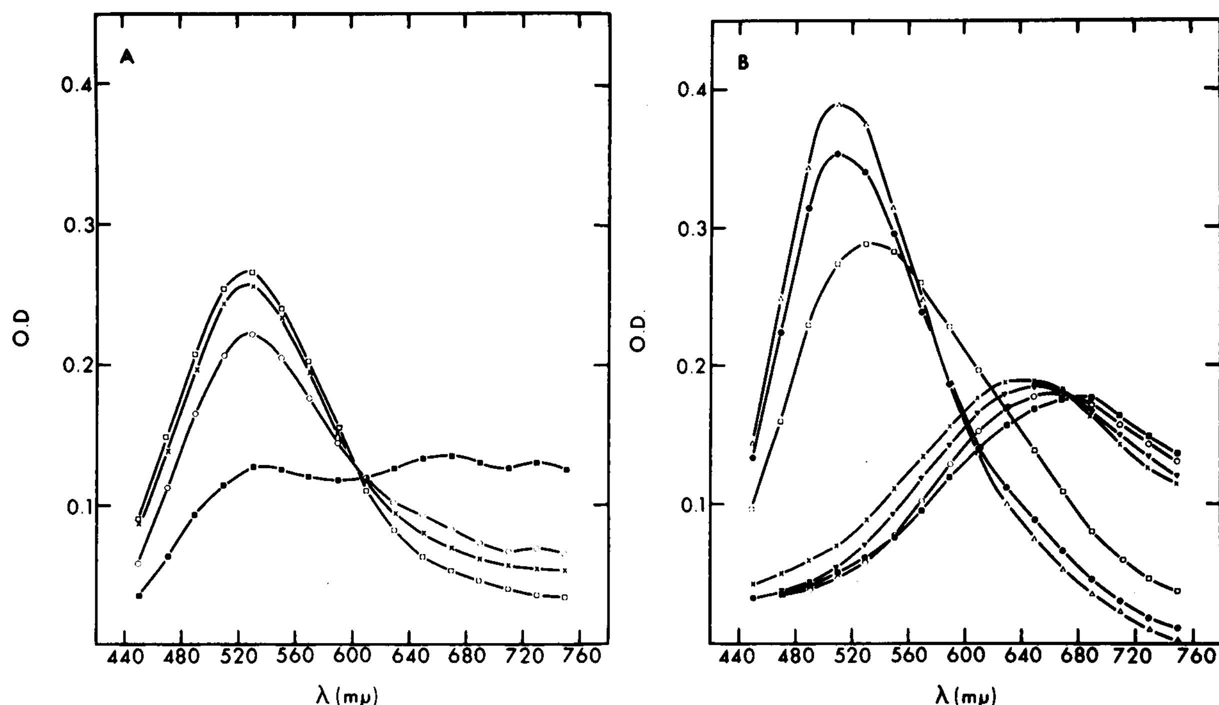

FIGURE 1. Visible spectra of HSA–Cu(II) (A) and DSA–Cu (II) (B): ■, pH 5.5; ○, pH 6.5; ▼, pH 7.6; ×, pH 8.0; □, pH 10; ●, pH 10.6; △, pH 11.6. Reprinted with permission from ref 5. Copyright 1971 Journal of Biological Chemistry.

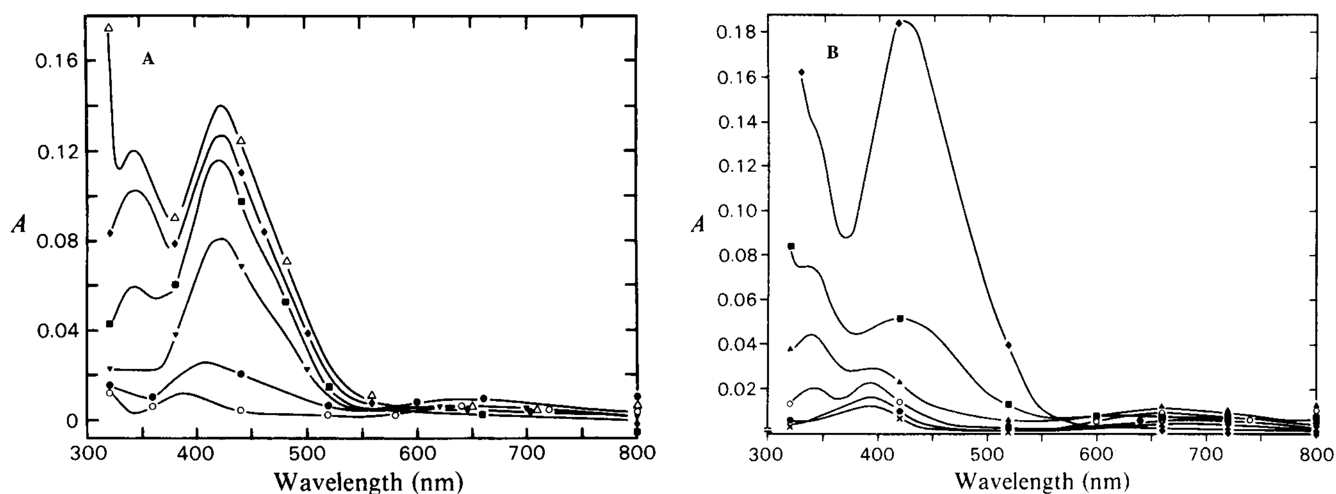

FIGURE 2. Visible spectra of HSA–Ni(II) (A) (○, pH 5.34; ●, pH 6.36; ▼, pH 6.94; ■, pH 8.19; ◆, pH 9.19; △, pH 10.14) and DSA–Ni(II) (B) (×, pH 5.43; ●, pH 6.09; ○, pH 7.31; ▼, pH 8.17; ■, pH 9.05; ◆, pH 10.21). Reprinted with permission from refs 12 and 13. Copyright 1982 Portland Press.

albumin–Cu(II), Cu(II)–histidine, and the ternary complex albumin–Cu(II)–histidine.[25] The studies of this equilibrium led to the development of copper–histidine therapy for Menkes disease, a fatal genetic disorder of copper transport.[26–28]

The structure of the main Cu(II)-binding site on bovine serum albumin (BSA) was first proposed by Peters and Blumenstock in 1967 as involving the α-amino nitrogen, two intervening peptide nitrogens, and the imidazole nitrogen.[29] Studies done in this laboratory such as equilibrium dialysis, visible spectroscopy, and proton displacement demonstrated the presence of a single high-affinity Cu(II)-binding site on human serum albumin (HSA).[4,25] The visible spectra of albumins and other peptides which may have the ATCUN motif provide a simple method of detection of the motif. In Cu(II)-binding studies, an absorption maximum at 525 nm which is reached at a low pH (pH 6.5 in HSA) and is maintained through a wide range of pH is indicative of a specific ATCUN motif (Figure 1A). Proteins which do not possess the motif demonstrate a broad maximum in the region of 600 nm which eventually migrates to 520 nm at very high pH (pH > 10) (Figure 1B).[5] The spectra of Ni(II) bound to the ATCUN motif show a peak at 420 nm at neutral pH which is characteristic of planar coordination (Figure 2A).[12] Nonspecific Ni(II)-binding is octahedral (Figure 2B).[13]

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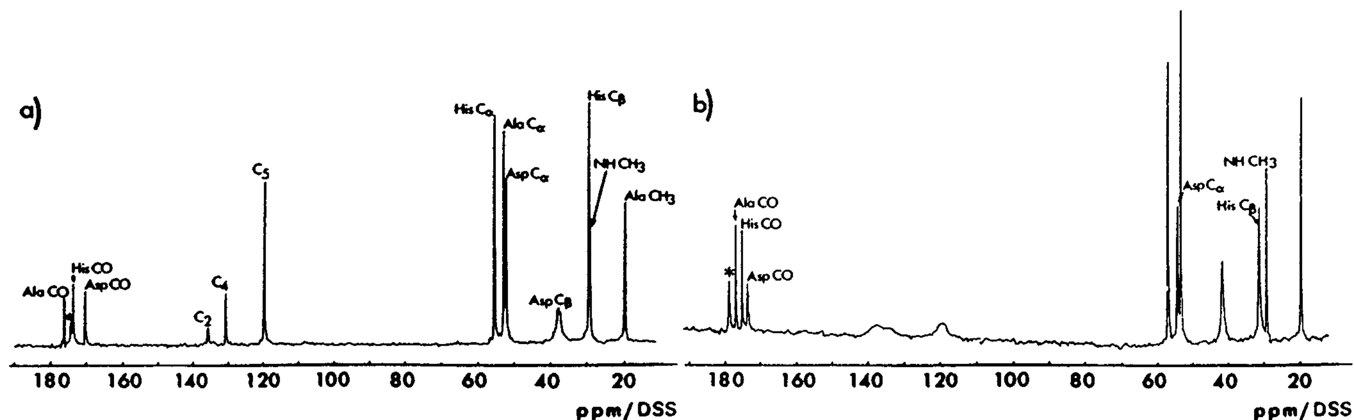

FIGURE 3. ^{13}C NMR spectra of Asp-Ala-His-Cu(II) in D_2O: (a) pH 2.0 and (b) pH 7.0. Reprinted with permission from ref 9. Copyright 1980 Journal of Biological Chemistry.

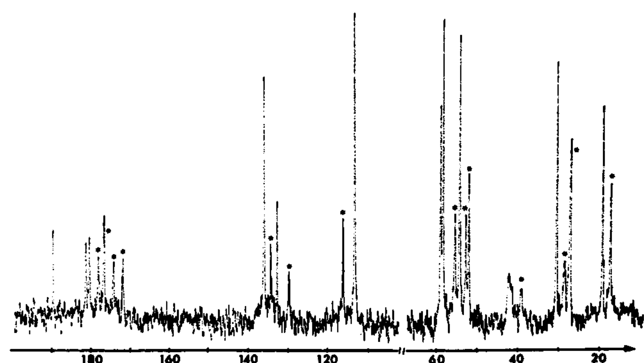

FIGURE 4. ^{13}C NMR spectra of Asp-Ala-His-Ni(II) in D_2O at pH 7.0. The peaks with an asterisk are free peptide; the peaks without an asterisk are Ni(II)−peptide. Reprinted with permission from ref 10. Copyright 1980 National Research Council of Canada.

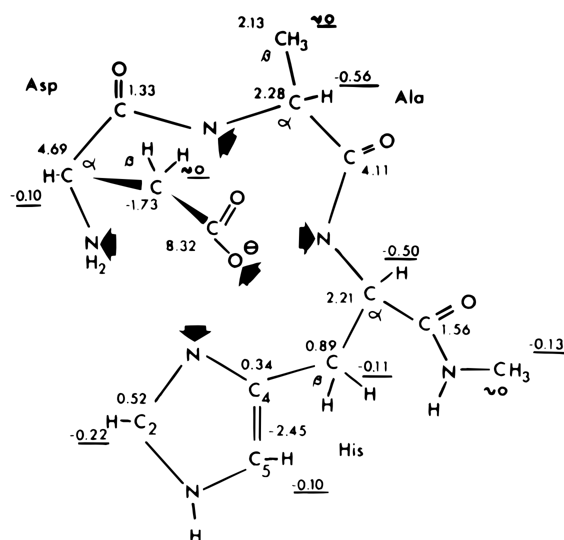

FIGURE 5. Chemical shifts ($\Delta = \delta_{complex} - \delta_{free\ peptide}$) for Asp-Ala-His-Ni(II) complex in D_2O at pH 9.1. Underlined values represent ^1H values and those not underlined are ^{13}C values (ppm). Arrows point to the site of Ni(II)-binding. Reprinted with permission from ref 10. Copyright 1980 National Research Council of Canada.

The Cu(II)- and Ni(II)-binding to the NH_2-terminal of HSA was studied in this laboratory by ^1H- and ^{13}C-NMR using the native sequence peptide Asp-Ala-His and the 24-residue peptide fragment from the NH_2-terminus of HSA.[9,10,15] Coordination of Cu(II) with the peptide showed a large line broadening for several ^{13}C resonances (especially C_2, C_4, and C_5 of the histidine, aspartyl C_β, aspartyl carboxylate, and histidyl C_β (Figure 3)). The results strongly suggested that, in addition to the four nitrogen ligands in a square planar coordination, there is also the involvement of the side chain carboxyl group, forming a pentacoordinated structure.[9,15] Similarly, the structure of the Ni(II)-binding site was studied by both ^{13}C- and ^1H-NMR spectroscopy (Figures 4 and 5).[10] The aspartyl COO^- carbon is most affected by Ni(II)-binding ($\Delta = 8.32$ ppm at pH 9.1) which is consistent with carboxylate−Ni(II) coordination. In the ^1H-NMR experiments, many upfield chemical shifts are observed for the imidazole residue as well as the C_α protons. Most significantly in DMSO-d_6 solution containing Ni(II)-Asp-Ala-His, there is a complete disappearance of the alanyl and histidyl NH protons which confirms the coordination of these two peptide nitrogens. These results also suggest that Ni(II) is complexed in a pentacoordinated structure. Similar results were obtained with peptide 1−24 of HSA.[15] The relevance of the carboxylate to metal affinity cannot be discerned readily from the available data. Direct evidence of the involvement of four in-plane nitrogen atoms was obtained by S-band ESR spectroscopy (Figure 6).[16]

Experiments with albumins of other mammalian species such as dog serum albumin (DSA) and chicken serum albumin (CSA) have demonstrated that substitution of the histidine residue in the third position causes a lack of specific binding of Cu(II). In DSA, tyrosine is substituted for histidine (Table 1).[5,6] The loss of the high-affinity Cu(II)-binding site in DSA has been correlated with the fact that dogs have high copper concentrations in the liver and are much more susceptible to copper toxicity than other species.[30] CSA has the NH_2-terminal sequence Asp-Ala-Glu-His (Table 1).[31] CSA is also unable to bind Cu(II) or Ni(II) specifically.[22]

The X-ray crystal structure of albumin demonstrates no defined structure at the amino terminal region of the protein.[35] Therefore, this region may have a relatively

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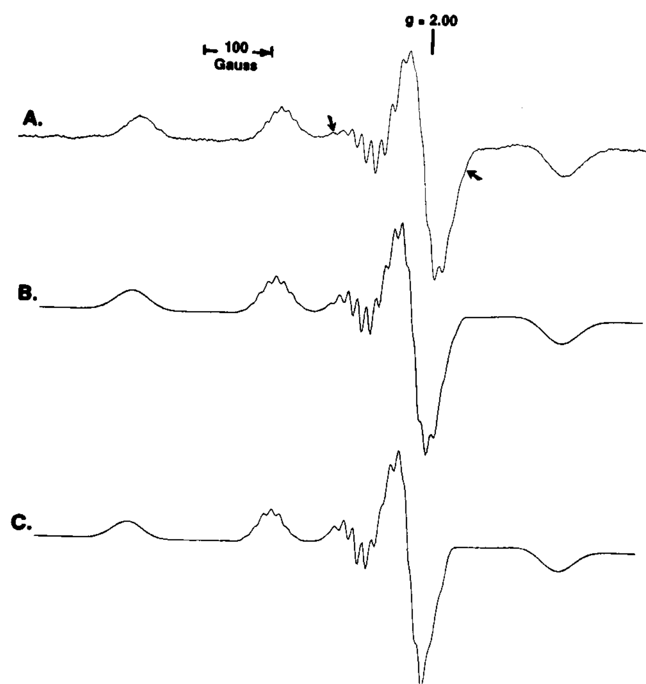

FIGURE 6. S-band ESR spectra of BSA−Cu(II) at pH 7.13: (A) experimental; computer simulation (B) assuming four equivalent nitrogens and (C) assuming three equivalent nitrogens. Reprinted with permission from ref 16. Copyright 1985 Elsevier Science.

Table 1. Sequences of Albumin NH_2-Termini, Human Sperm Protamine P2a, Histatin 3 (Salivary Peptide), and Neuromedins[6,31−34,65,70,74] [a]

RSA	Glu	Ala		His	Lys	Ser	Glu	Ile	Ala	His	Arg	Phe	Lys	Asp	Leu
HSA	Asp	Ala		His	Lys	Ser	Glu	Val	Ala	His	Arg	Phe	Lys	Asp	Leu
BSA	Asp	Thr		His	Lys	Ser	Glu	Ile	Ala	His	Arg	Phe	Lys	Asp	Leu
PSA	Asp	Thr		Tyr	Lys	Ser	Glu	Ile	Ala	His	Arg	Phe	Lys	Asp	Leu
DSA	Glu	Ala		Tyr	Lys	Ser	Glu	Ile	Ala	His	Arg	Tyr	Asn	Asp	Leu
CSA	Asp	Ala	Glu	His	Lys	Ser	Glu	Ile	Ala	His	Arg	Tyr	Asn	Asp	Leu
Human Sperm Protamine P2a	Arg	Thr		His	Gly	Gln	Ser	His	Tyr	Arg	Arg	Arg	His	Cys	Ser
Histatin 3	Asp	Ser		His	Ala	Lys	Arg	His	His	Gly	Tyr	Lys	Arg	Lys	Phe
Neuromedin C	Gly	Asn		His	Trp	Ala	Val	Gly	His	Leu	Met	NH_2			
Neuromedin K	Asp	Met		His	Asp	Phe	Phe	Val	Gly	Leu	Met	NH_2			
Neuromedin B	Gly	Asn		Leu	Trp	Ala	Thr	Gly	His	Phe	Met	NH_2			

[a] ATCUN motifs are underlined.

flexible conformation, resulting in the motif being able to fold around a metal to form the specific binding site for Cu(II) and Ni(II).

Design of the ATCUN Motif

The NH_2-terminal protein sequences of albumins from various species (Table 1) were utilized for the initial design of the ATCUN motif.[36−38] BSA, rat serum albumin (RSA), and HSA have a functionally very similar strong and specific binding site for Cu(II) at the NH_2-terminus, whereas DSA lacks this specific site.[5,6] This indicated that the NH_2-terminal aspartyl residue in HSA and BSA may not be absolutely essential for formation of the ATCUN motif. RSA also binds one Cu(II) specifically, although it has a glutamyl residue at the NH_2-terminus. It also did not appear to make much difference which amino acid residue is in the second position given that there are two intervening peptide nitrogens. HSA and BSA have Ala and Thr, respectively, in the second position, and yet both bind one Cu(II) specifically with similar binding characteristics. With this rationale, any amino acid with the exception of a proline residue in the second position should fulfill the criteria necessary for metal-binding. However, the histidine residue in position 3 did appear to be mandatory since HSA, BSA, and RSA which have histidine in position 3 bind Cu(II) specifically whereas DSA which has a tyrosine in position 3 lacks the specific Cu(II)-binding site. These features, as well as the geometry of the ligand array in the native protein, and the nature of the donor groups, were considered in the design of Gly-Gly-His (in *N*-methyl amide form to mimic the protein), the first design using the ATCUN motif.

Table 2. Comparison of Log Stability Constants (log β'_{pqr} and log β''_{pqr}) of the Complex Species $M_pH_qA_r$ [M = Cu(II), H = hydrogen ion, A' = Gly-Gly-His-*N*-methyl amide, A'' = Asp-Ala-His-*N*-methyl amide] in 0.15 M NaCl at 25 °C[7,14]

p	q	r	log β'_{pqr} ($M_pH_qA'_r$)	log β''_{pqr} ($M_pH_qA''_r$)
0	3	1		17.267
0	2	1	14.47	14.286
0	1	1	8.00	7.31
1	−2	1	−0.479	−0.55

The model building showed the designed molecule to have the required square planar coordination geometry and peptide backbone configuration. Furthermore, we analyzed the conformation of Gly-Gly-His theoretically and compared it with that of Asp-Ala-His using energy minimization (Figure 7).[37,38] The calculations showed that several folded conformations were possible for a square planar complex involving the amino terminal nitrogen, two peptide nitrogens, and the imidazole nitrogen (Figure 8). The peptide was synthesized and the speciation analyses, stability constants of the species, and the visible and ESR spectral properties of the Cu(II)-Gly-Gly-His complex showed properties similar to those of the ATCUN motif of HSA (Tables 2 and 3), (Figure 9).[5,7,11,14,16,25,36] The structure of Cu(II)-Gly-Gly-His was solved by X-ray crystallography.[39] The Cu(II) is tetradentate, chelated by the NH_2-terminal nitrogen, the next two peptide nitrogens, and a histidyl imidazole nitrogen of Gly-Gly-His in a slightly distorted square planar arrangement (Figure 10).

DNA Cleavage by the ATCUN Motif

After the structural characterization of Cu(II)-Gly-Gly-His, Pauling and co-workers[40] showed that this complex had antitumor activity. A Cu(II)-Gly-Gly-His/ascorbate system was able to kill Ehrlich ascites tumor cells *in vitro*. Mice

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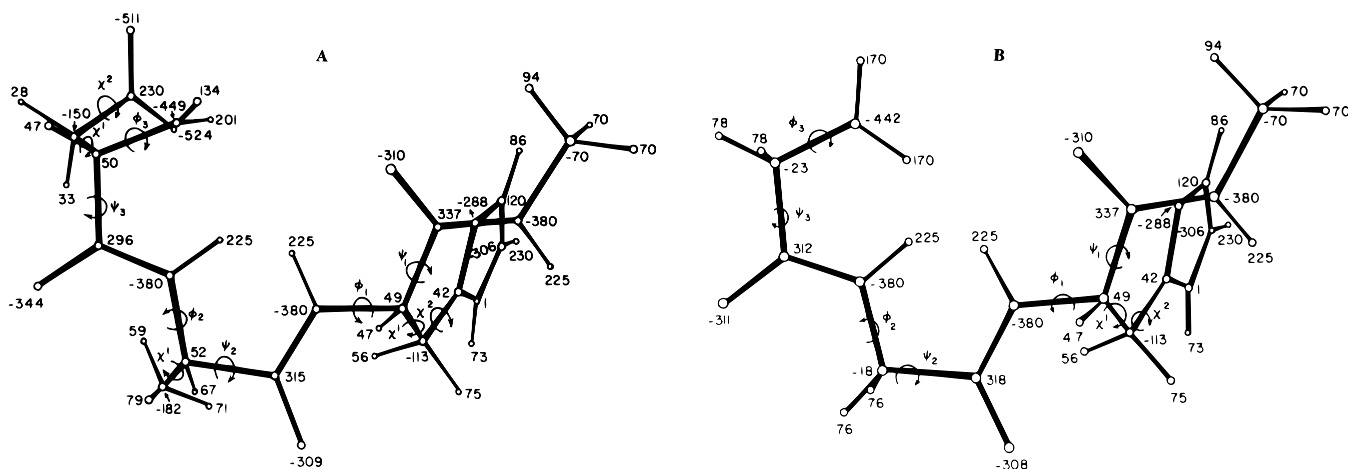


FIGURE 7. Perspective of an extended conformation showing the definition of torsion angles and the *ab initio* (STO-3G) monopole charges in 10^3 electron units: (A) Asp-Ala-His, (B) Gly-Gly-His. Reprinted with permission from ref 38. Copyright 1977 Kluwer Academic Publishers.

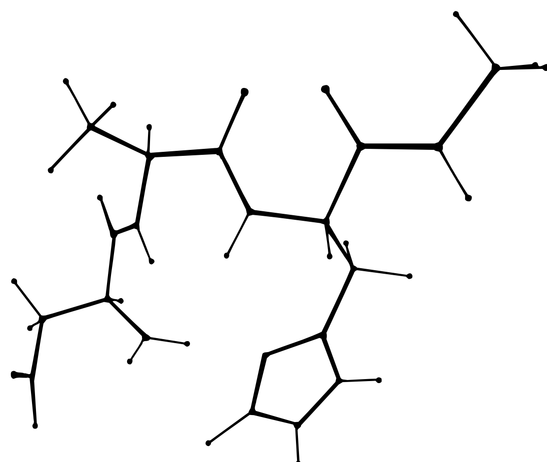


FIGURE 8. One of the plausible folded conformations of Asp-Ala-His. Similar conformations were also noted for Gly-Gly-His to provide an array of four nitrogens in an approximately square planar geometry for Cu(II) binding. Reprinted with permission from ref 38. Copyright 1977 Kluwer Academic Publishers.

Table 3. Comparison of Visible and ESR Spectral Properties of Cu(II) Complexes at pH 6.5[5,7,11,14,16,25,36]

complex	λ_{max} (nm)	ϵ_{max} ($mol^{-1} \cdot L \cdot cm^{-1}$)	g_{11}	g_m	A_{11} (G)	$A_{11}{}^a$ (mk)
HSA−Cu(II)	525	101	2.166	2.051	214	21.6
Gly-Gly-His-Cu(II)	525	103	2.170	2.051	211	21.3
Asp-Ala-His-Cu(II)	525	103	2.167	2.050	211	21.4
DSA−Cu(II)	600 (br)		2.256	2.059	163	17.2

[a] A (mk) = 0.046686gA (G).

which were inoculated with the same cells were shown to have a longer life span when they were treated with a Cu(II)-Gly-Gly-His/ascorbate system. Although Cu(II) is much less efficient in oxidation when it is bound to protein ligands such as the ATCUN motif than when it is free, a significant amount of reactivity still exists. The ATCUN motif-bound Cu(II) is hypothesized to cleave DNA or proteins by the production of hydroxyl radicals:[41]

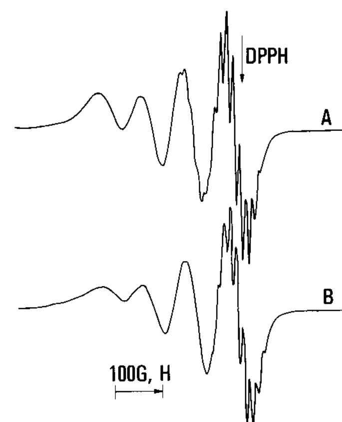
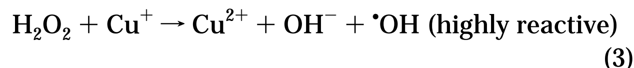


FIGURE 9. ESR spectra of (A) Gly-Gly-His-Cu(II) at pH 6.4 and (B) Asp-Ala-His-Cu(II) at pH 6.5. Reprinted with permission from ref 11. Copyright 1981 Elsevier Science.

$$\text{ascorbate} + 2Cu^{2+} \rightarrow \text{dehydroascorbate} + 2Cu^{+} + 2H^{+} \quad (1)$$

$$\text{ascorbate} + O_2 \xrightarrow{Cu^{2+}} \text{dehydroascorbate} + H_2O_2 \quad (2)$$



$$H_2O_2 + Cu^{+} \rightarrow Cu^{2+} + OH^{-} + {}^{\bullet}OH \text{ (highly reactive)} \quad (3)$$

Hydroxyl radicals (${}^{\bullet}OH$) can react with a DNA backbone and cleave the DNA strand. Therefore, in the presence of ascorbate, ATCUN motif bound to Cu(II) can produce ${}^{\bullet}OH$ which in turn can cleave DNA. ATCUN motif-bound Cu(II) may be able to produce superoxide anions (O_2^-) which can then combine with H_2O_2 to produce ${}^{\bullet}OH$:[42]

$$H_2O_2 + O_2^{-} \rightarrow O_2 + OH^{-} + {}^{\bullet}OH \quad (4)$$

Both superoxide anions and hydrogen peroxide are produced *in vivo*. Superoxide anions are released from hemoglobin-bound O_2 at a rate of 10^3 to 10^6 molecules per erythrocyte per second.[43] Superoxide dismutase converts superoxide to H_2O_2 and O_2.[43,44] Therefore, Cu(II) bound to an ATCUN motif should be able to cleave DNA or proteins *in vivo* as well as *in vitro*.

As with Cu(II), the chemistry of peptide- or protein-bound Ni(II) is different from that of unbound Ni(II). ATCUN motif-bound Ni(II) is capable of DNA cleavage

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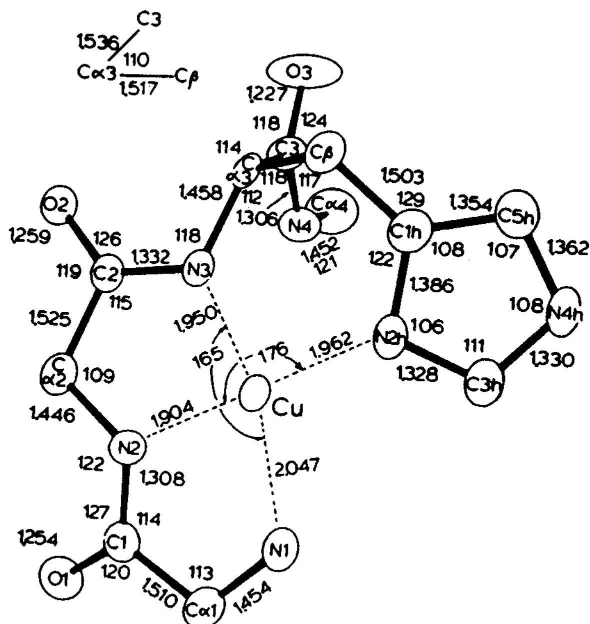

FIGURE 10. Structure of the Cu(II) complex of the designed peptide Gly-Gly-His-*N*-methyl amide by X-ray crystallography. Reprinted with permission from ref 39. Copyright 1976 National Research Council of Canada.

but cleaves DNA using a different chemistry than ATCUN motif-bound Cu(II). Nickel undergoes redox cycles between Ni(III) and Ni(II) when it is bound to peptides and proteins. It has been shown that Ni(III) compounds have oxygen radical character.[44−46] These differing redox properties result in nickel being unable to produce DNA cleavage in the presence of ascorbate. However, ATCUN motif-bound Ni(II) is still able to cleave DNA through the production of hydroxyl radicals and superoxide anions in the presence of H_2O_2.[47] ATCUN motif-bound Ni(II) is also able to cause protein cross-linking.[48] However, this activity does not appear to be mediated by any oxygen-containing intermediates. Aromatic amino acids appear to be the targets of the cross-linking.

Protein Design Utilizing the ATCUN Motif

The design of proteins is a relatively new field. Several groups have used synthetic peptide scaffolds onto which metal-binding sites are designed.[49,50] However, the use of preexisting metal-binding motifs in hybrid proteins allows a designed protein to have the combined properties of two or more proteins. The simplicity of the ATCUN motif has led to its use in several protein and peptide designs.

Dervan and co-workers[51−53] designed a DNA cleavage protein to utilize the ability of Cu(II)- or Ni(II)-Gly-Gly-His to cleave DNA. They took the NH_2-terminus of the DNA-binding region of Hin recombinase (Hin(139−190)), a helix−turn−helix protein and attached Gly-Gly-His to the NH_2-terminal end through solid phase synthesis to produce Cu(II)-Gly-Gly-His-Hin(139−190). Their experiments with the Cu(II)-bound form of the protein demonstrated some cleavage of DNA. Experiments with the Ni(II)-bound form of the protein showed much more significant DNA cleavage. Gly-Gly-His has also been designed to be placed at the NH_2-terminus of Sp1, a zinc finger protein.[54] However, the Zn(II) atoms in zinc fingers, including Sp1, can be replaced by other metals including Ni(II).[55−57] Copper(II) or Ni(II) bound at another site, including within the zinc finger motif of Sp1, could produce specific cleavage of the DNA. Recent studies of iron fingers show such a cleavage.[57]

A design by Long and co-workers[58] uses a modified ATCUN motif which does not clearly require placement at the NH_2-terminus. This uses the amino acid ornithine which has a simple amino group as its R group. They showed that the Cu(II)- and Ni(II)-bound forms of NH_2-Tyr-Ala-(δ)-Orn-Gly-His-$CONH_2$ could cleave DNA in a similar manner as the Cu(II)-Gly-Gly-His-Hin(139−190). The metal-binding characteristics of this peptide were well documented. Specific DNA cleavage has also been shown by an analog of netropsin containing Gly-Gly-His.[59]

We have recently designed and expressed a protein, Gly-Lys-His-Fos(138−211), which is capable of binding specifically to an AP-1 binding site containing DNA and cleaving DNA specifically.[60] We designed the motif peptide, Gly-Lys-His, on the basis of our original design of Gly-Gly-His.[36−39] The design places this ATCUN motif at the amino terminal end of the basic domain of Fos (Fos (138−211)). This segment of Fos contains the basic and leucine zipper regions only. Jun(248−334), which contains the basic and leucine zipper regions of that protein, was used to heterodimerize with Gly-Lys-His-Fos(138−211). Fos homodimers do not form as readily as Jun homodimers. Therefore, by designing the ATCUN motif on Fos rather than Jun, it would be more likely that only one active protein would be present in each dimer. Molecular modeling utilizing X-ray structure coordinates of Cu(II)-Gly-Gly-His[39] and using the program INSIGHT showed that an ATCUN motif with lysine in the second position should form a structure similar to Gly-Gly-His. The positively charged lysine residue in the second position from the NH_2-terminus should also increase the affinity of the motif for DNA due to electrostatic interactions with the negatively charged phosphate backbone. The lysine would also increase the α-helicity of the protein and therefore increase the propensity for binding to the

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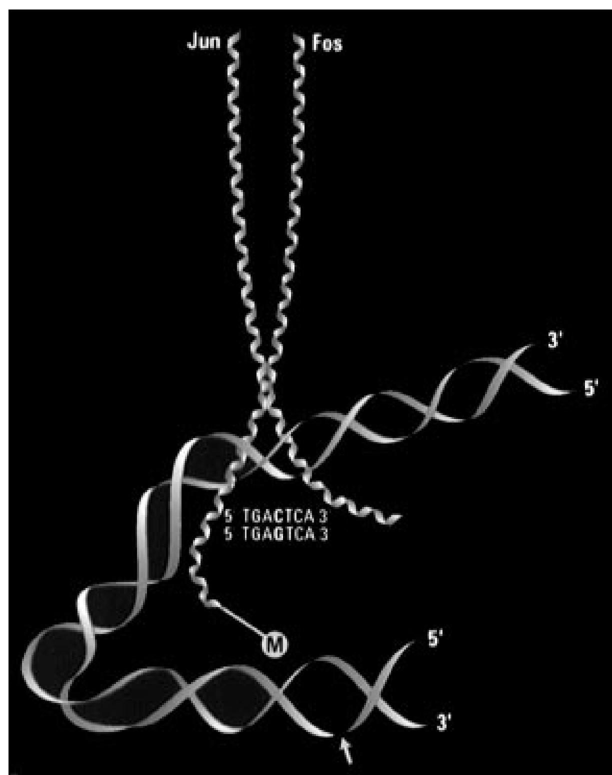

FIGURE 11. A composite diagram of DNA cleavage by M(II)-Gly-Lys-His-Fos(138−211). Jun(248−334) (blue) is shown complexed to M(II)-Gly-Lys-His-Fos(138−211) (red). Both are complexed to DNA (green). The metal (yellow) can be either Cu(II) or Ni(II). DNA cleavage occurs slightly more than three helical turns upstream of the AP-1-binding site. The lysine and copper in the designed motif itself may bring the DNA toward the motif via a bend as diagrammed, causing specific cleavage. Reprinted with permission from ref 60. Copyright 1996 American Chemical Society.

major groove of DNA.[61] Therefore, a lysine residue should promote DNA-binding on the basis of charge and of secondary structure. ATCUN motif tripeptides with positively charged residues have recently been shown to affect the site of DNA cleavage.[62]

We expressed the proteins in *Escherichia coli.* Gly-Lys-His-Fos(138−211) was shown to cleave several forms of DNA, including circular and linear segments 100−1900 base pairs (bp) long. The cleavage is specific for a site 31−33 bp upstream from the AP-1-binding site (Figure 11).[60] Both monoperoxyphthalic acid and the hydrogen peroxide/ascorbate system were shown to be capable of catalyzing the cleavage by Cu(II) or Ni(II). The cleavage in each case was shown to be single-stranded, indicating that the interaction between the cleavage motif and the DNA was very specific.

The X-ray crystal structure of c-Fos and c-Jun binding to the AP-1-binding site containing DNA has recently been published.[63] Our cleavage results gave further information on the orientation of Fos(138−211) on DNA. They indicate that the NH_2-terminus of Gly-Lys-His-Fos(138−211) is only found at the 5′ end of the AP-1-binding site. The lack of intervening cuts between the AP-1-binding site and the cleavage site upstream indicated that the DNA was bent by the binding of the two proteins so that the region which is slightly more than three turns upstream is brought back toward the AP-1-binding site (Figure 11). This may be due to bending by the Fos-Jun heterodimer.[64] In addition, the positively charged lysine residue and copper atom within the ATCUN motif also induce the DNA backbone upstream of the ATCUN motif to curve back toward the AP-1-binding site.

We have designed a protein which is capable of cleaving DNA. Once expressed, this protein retains the DNA-binding capabilities of its parent bZIP protein, Fos, as well as the metal-binding properties of the ATCUN motif. The DNA-binding moiety brings the DNA cleavage moiety into close proximity to the DNA to increase the specificity of the DNA cleavage. Factors which may influence the site of cleavage include the chemical characteristics of any side chain in or near the motif and chirality of the amino acids.[62] These studies have demonstrated that the definition of the ATCUN motif can be used to design proteins which have properties beyond the minimal Gly-Gly-His motif.

Prediction of the ATCUN Motif in Other Proteins

The definition of the ATCUN motif has already enabled us to predict from their primary sequences that several unrelated proteins and peptides possessed this site (Table 1). Neuromedins C and K possess ATCUN motifs.[65,66] This property may have significant implications for the function of these neurotransmitters. There may be adverse effects involving the neurotransmission function of neuromedin C in the Cu(II) deficiency state (Menkes disease) and/or toxic overload of Cu(II) (Wilson disease).[67] Neuromedin C and/or its longer form, gastrin releasing peptide, can act as growth factors for small cell lung, prostate, and pancreatic cancers.[68,69] The copper-binding ability of neuromedin C may affect its function as a growth factor. The definition of the ATCUN motif may also be used to predict specific Cu(II)- and Ni(II)-binding sites in other proteins and peptides. A data base search of protein sequences has revealed that human sperm protamine P2a has an ATCUN motif (Arg-Thr-His-).[70] A peptide representing this motif has recently been synthesized. It was found that Ni(II) and Cu(II) bound the peptide strongly and that the resulting complex mediated oxidative DNA damage.[71] This may lead to oxidative damage of sperm DNA in metal-exposed individuals which raises the possibility of cancer in the progeny and birth defects.

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Table 4. Unfavorable Conditions for the ATCUN Motif

condition	result
proline residues in first or second positions	lack of a peptide nitrogen bound hydrogen atom prevents binding by Cu(II) or Ni(II)
modified amino terminus (e.g., acetylated)	lack of free amino terminal nitrogen prevents binding by Cu(II) or Ni(II)
positively charged residues in the vicinity of the motif	these may destabilize the motif
steric hindrance	this may occur when the amino terminus containing a potential motif is buried within the protein's structure

Epidemiological studies have revealed significant correlations between paternal exposure to metals (welders, machinists, autobody repairmen, etc.) and incidences of childhood cancers.[72,73] Histatins are a family of 12 salivary peptides, 8 of which have histidine in the third position,[74] providing an ATCUN motif to bind Ni(II) and Cu(II).

However, certain caveats must be observed in the prediction of ATCUN motifs. According to the definition, two peptide nitrogens are necessary for a motif. This rules out the presence of a proline residue in the first or second position. The NH_2-terminus must be free; i.e., there can be no posttranslational modifications such as acetylation of the NH_2-terminus.

It has already been demonstrated that other residues may have some interactions with the metal atoms in the motif. Experiments with Asp-Ala-His demonstrated that the carboxylate group of the aspartate residue is bound to the metal atom due to ionic interactions.[15] In Gly-Lys-His-Fos(138−211) there are multiple positively charged lysine and arginine residues C-terminal to the ATCUN motif, as well as the positively charged lysine residue in the second position of the motif. The motif is still capable of metal binding at physiological pH. Residues with other charged side groups which are in the first, second, or potentially fourth or fifth position from the NH_2-terminus or nearby in the tertiary structure of the protein may interact with the motif. These may form destabilizing or stabilizing interactions depending upon the geometry of the interaction.

Finally, the NH_2-terminus must be accessible to metal atom binding. In small peptides such as neuromedin C, this is not a significant consideration, but in large polypeptides, the NH_2-terminal region may be buried within the tertiary structure of the protein. In this case, the metal may be unable to approach the site due to steric hindrance or because the conditions surrounding the NH_2-terminus are too hydrophobic unless the metal is bound prior to completion of protein folding. As well, once the metal has accessed the ATCUN motif site, the backbone of the ATCUN motif must be able to fold around the metal atom, which may not be possible because of steric hindrance from the rest of the protein. Situations in which formation of the ATCUN motif may not be favored are summarized in Table 4.

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Conclusions

The early work on the binding of Cu(II) and Ni(II) by albumins determined that there was a high-affinity Cu(II)- and Ni(II)-binding site at the NH_2-terminus of certain albumins, thus termed the NH_2-terminal Cu(II)- and Ni(II)-binding (ATCUN) site. NMR, ESR, visible spectroscopy, and X-ray crystallography determined that the metal was coordinated in this site in a square planar configuration by the α-NH_2-terminal nitrogen, the two intervening peptide nitrogens, and the imidazole nitrogen. Studies of albumins and model peptides with and without the high-affinity binding sites demonstrated the requirement of histidine in the third position from the NH_2-terminus for the formation of an ATCUN motif. Therefore, the ATCUN motif could be defined as an NH_2-terminal tripeptide where (1) the NH_2-terminus is free, (2) there is a histidine in the third position from the NH_2-terminus, and (3) there are two free intervening peptide nitrogens between the first and the third residues.

The comparison of protein sequences to the ATCUN definition led to the discovery that other unrelated proteins and peptides possessed this site and could bind metals. This previous unknown property of several proteins could have significant implications for their functions. The ATCUN site in human sperm protamine P2a in metal-exposed individuals may be a potential mechanism for carcinogenesis or birth defects in the offspring. Other proteins with this site may transport Cu(II) and Ni(II) or may damage other biomolecules. Thus, the ATCUN site may have much wider physiological relevance than merely being a metal transport site in albumins.

This motif can also be very versatile in protein design. It can generate novel proteins by expression which can cleave DNA or other proteins specifically. We can expect major advances in the application of such a design in delineating the site of specific protein−DNA or protein−protein interactions, site specific cutting of genomic DNA, and possibly cleaving specifically a stretch of aberrant DNA sequences inside a cell.

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