constant for ribonuclease S′ under conditions where P_2 and P* are insignificant. Assuming, however, that neither P* nor P_2 binds substrate, any equilibrium involving P, P*, and P_2 should be affected by the presence of substrate and the effect should appear as an apparent effect on S-peptide–S-protein binding.

Using the limited data given above, it is possible to estimate to what extent the different reactions in Scheme I influenced the different binding studies reported in the literature, and such a survey has been carried out in Table V. Clearly this qualitative comparison is only approximate, but it may nevertheless serve as a first step toward a definition of all the variables that ultimately will have to be described in order to understand the S-protein–S-peptide interaction.

At present it is clear that the experimental conditions under which meaningful data on reaction 5 can be obtained will have to be restricted until more quantitative data are available for all the remaining seven reactions. It appears, however, that low-temperature, low S-protein concentration, and absence of substrate contribute a good deal toward simplifying the system, and these are the conditions under which our results were obtained.

Acknowledgment

The authors express their thanks to Miss Adella Grevious for her technical assistance and to Drs. Wayne Bolen and Rufus Lumry for their help with the calorimeter studies.

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Proton Nuclear Magnetic Resonance Studies of the Association of Ribonuclease S-Peptide and Analogs with Ribonuclease S-Protein*

F. M. Finn, J. Dadok, and A. A. Bothner-By†

ABSTRACT: The 250-MHz proton nmr spectra of D_2O solutions of S-peptide and a number of its subfragments and analogs have been recorded. The binding of S-peptide and its analogs to S-protein is accompanied by an upfield shift of the histidine-12 ring proton signals and a broadening and diminution in intensity of the signals from the arginine-10 δ protons and the methionine SCH_3 protons. These observations are in accord with a model of binding which postulates a random-coil conformation for the free S-peptide and the formation of a salt-bridge link between glutamate-2 and arginine-10 on binding. The findings are consistent with the structures deduced for ribonuclease S by X-ray diffraction.

Bovine pancreatic ribonuclease A has been extensively investigated from the point of view of structure and function. The primary amino acid sequence was determined by Smyth *et al.* (1963). X-Ray diffraction studies of crystalline ribonuclease A have been conducted by Kartha *et al.* (1967), who have refined the three-dimensional structure to 2 Å. Cleavage of a single peptide bond between alanine-20 and serine-21 may be brought about by hydrolysis with subtilisin under mild conditions (Richards, 1958). The products, S-peptide and S-protein, combine in solution to form a 1:1 complex, ribonuclease S, with the full enzymatic activity of the original ribonuclease, as demonstrated by Richards (1958). Crystalline ribonuclease S has been studied by X-ray diffraction by Wyckoff *et al.* (1970), who have established its structure to 2-Å resolution. The dissociation constant of ribonuclease S has been measured using various methods and has been assigned rather widely varying values, from 7×10^{-5} M (Woodfin and Massey, 1968; Moroder *et al.*, 1971) to 1×10^{-8} M (Kershaw and Richards, 1966; Hearn *et al.*, 1971; Berger and Levit, 1971). The latter value was measured in the pres-

* From the Protein Research Laboratory, University of Pittsburgh, School of Medicine (F. M. Finn), and the Mellon Institute of Science, Carnegie-Mellon University, Pittsburgh, Pennsylvania (J. D. and A. A. B.-B.). *Received September 15, 1971.* This work was performed using the NMR Facility for Biomedical Research supported by the National Institutes of Health Grant RR 00292.

† Author to whom correspondence should be addressed.

TABLE I: Amino Acid Sequences of S-Peptide and Analogs.

Compound	Name	
1	S-Peptide	Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His -Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala
2	S-Peptide$_{1-14}$	Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His -Met-Asp
3	[12-β-Pyrazolylalanine]-S-peptide$_{2-14}$	Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-Bpa[a] -Met-Asp
4	[12-β-Pyrazolylalanine]-S-peptide$_{3-14}$	Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-Bpa[a] -Met-Asp
5	[12-(3-Carboxymethylhistidine)]-S-peptide$_{1-14}$	Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-Cmh[b]-Met-Asp
6	[10-Ornithine,12-(3-carboxymethylhistidine)]-S-peptide$_{1-14}$	Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Orn-Gln-Cmh[b]-Met-Asp

[a] β-(3-Pyrazolyl)alanine. [b] 3-Carboxymethylhistidine.

ence of cytidine 2′,3′-phosphate as a substrate, which may increase the binding by formation of an enzyme–substrate complex (Markus *et al.*, 1968). (The sequences of S-peptide and the analogs used in this study are shown in Table I.)

The X-ray diffraction studies of ribonuclease S define certain aspects of the binding of S-peptide rather clearly. In the complex the S-peptide contains three turns of helix involving residues threonine-3 through methionine-13. Backbone H bonding to S-protein can occur between the carbonyl of histidine-12 and the amide of valine-47 and between the carbonyls of valine-47 and His-48 and the amide hydrogens of aspartic acid-14 and serine-16, respectively, serine-16 being especially well defined in the electron density diagrams. Valine-47, leucine-51, and valine-54 cooperate to produce a hydrophobic pocket to which the methionine-12 side chain may bind. The arginine-10 side chain is bent back toward the amino-terminal end of the peptide; however the positions of lysine-1 and glutamic acid-2 are very poorly defined. The imidazole ring of histidine-12 is positioned about 5 Å from the benzene ring of phenylalanine-8, though the plane of the imidazole ring is not well fixed.

The nature of the binding and the structural features nec-

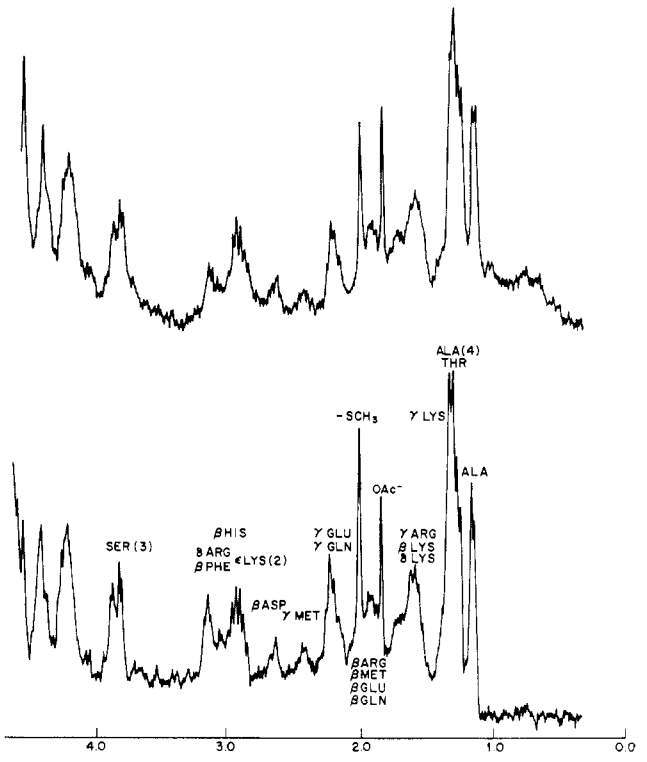

FIGURE 1: S-Peptide, 2% in D_2O. Lower trace: without S-protein. Upper trace: 0.1 equiv of S-protein was added.

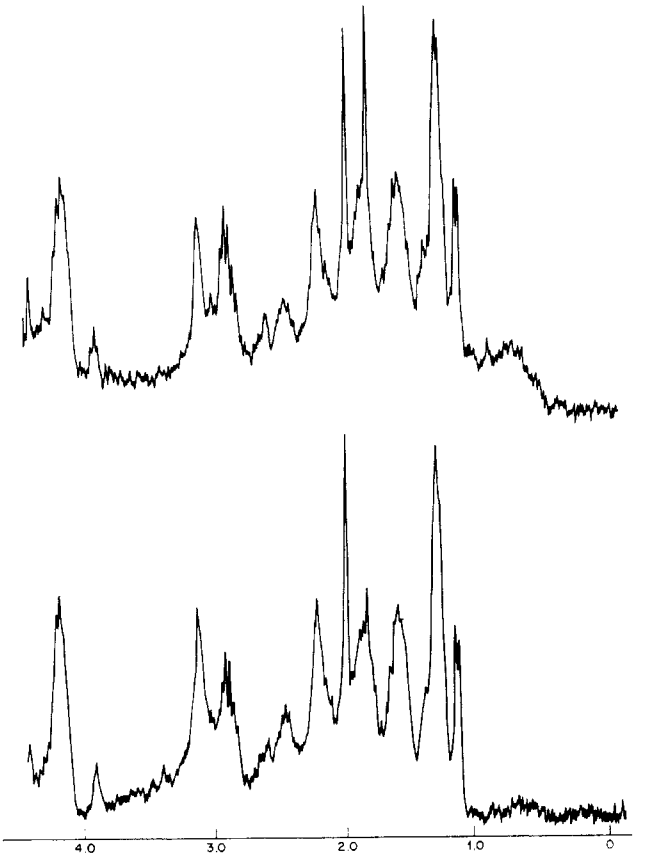

FIGURE 2: S-Peptide$_{1-14}$, 2% in D_2O. Lower trace, in absence of S-protein. Upper trace 0.08 equiv of S-protein was added.

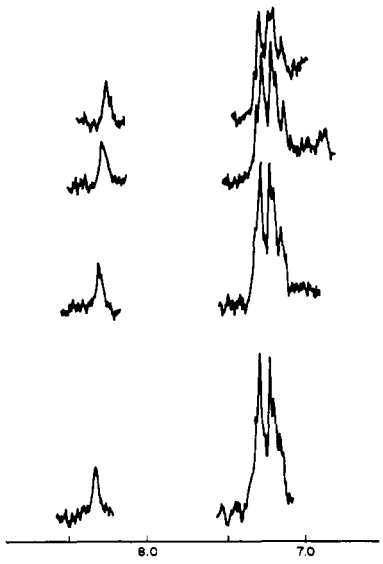

FIGURE 3: Aromatic region, S-peptide, 2% in D_2O. Lower trace, in absence of S-protein. Upper traces 0.1, 0.2, 0.4 equiv of S-protein was added.

essary for enzymatic activity have also been studied by chemical modification techniques. For example, it has been shown that a shortened S-peptide containing only residues 1–14 binds to S-protein equally as well as the full 20-peptide fragment, and that the complex possesses full enzymatic activity (Hofmann *et al.*, 1966a,b).

In an attempt to obtain further information about the details of the process whereby S-peptide binds to S-protein, we have examined the 250-MHz proton nuclear magnetic resonance (nmr) spectra of synthetic S-peptide and some synthetic analogs thereof, and have observed changes in the spectra upon the addition of 0.05–0.40 equiv of protein. The rationale for this procedure is as follows.

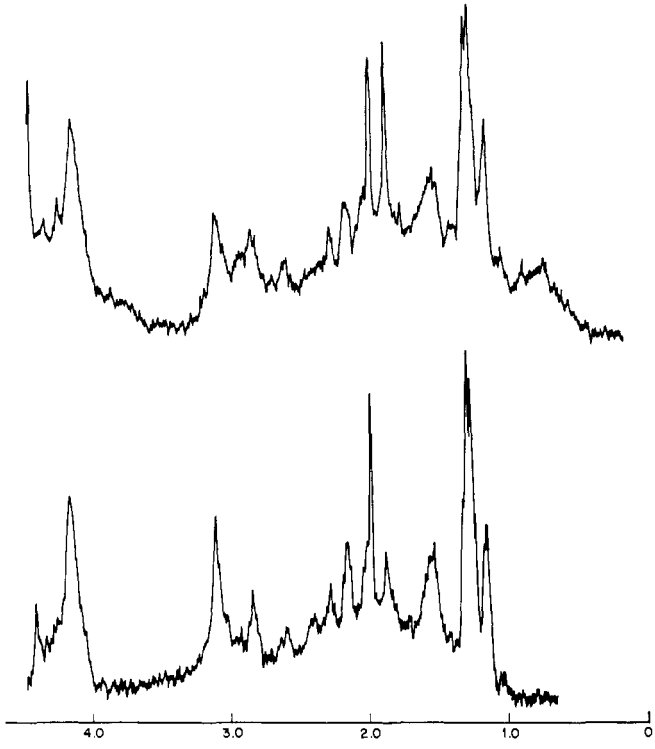

FIGURE 4: [12-β-Pyrazolylalanine]-S-peptide$_{2-14}$, 2% in D_2O. Lower trace in absence of S-protein. Upper trace: 0.08 equiv of S-protein was added.

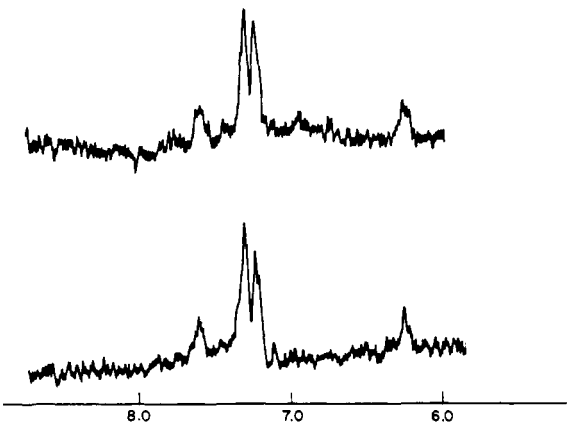

FIGURE 5: Aromatic region, [12-β-pyrazolylalanine]-S-peptide$_{2-14}$. Lower trace: in absence of S-protein. Upper trace: 0.05 equiv of S-protein was added.

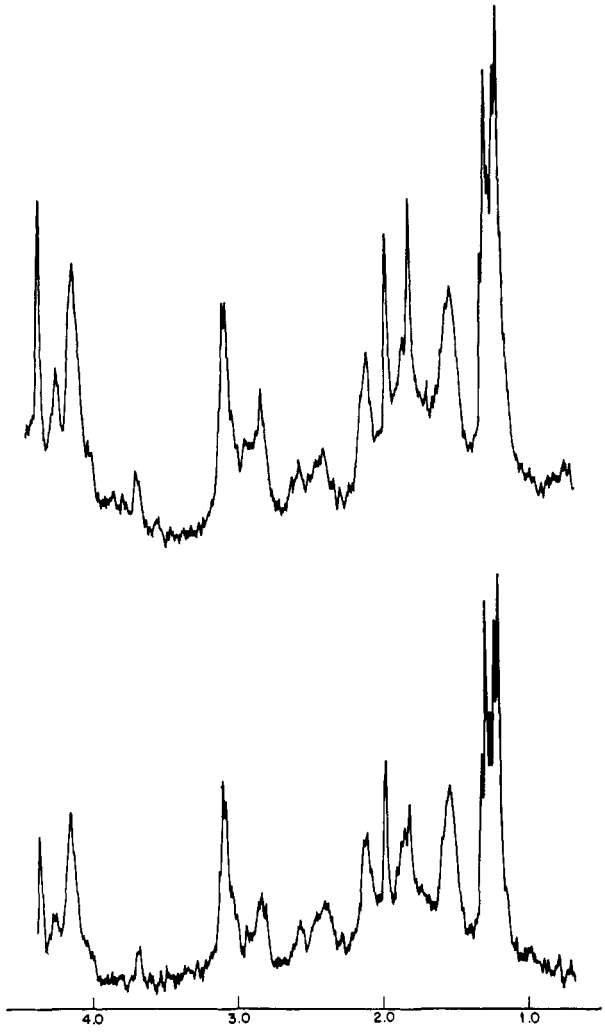

FIGURE 6: [12-β-Pyrazolylalanine]-S-peptide$_{3-14}$, 2% in D_2O. Lower trace: in absence of S-protein. Upper trace: 0.05 equiv of S-protein was added.

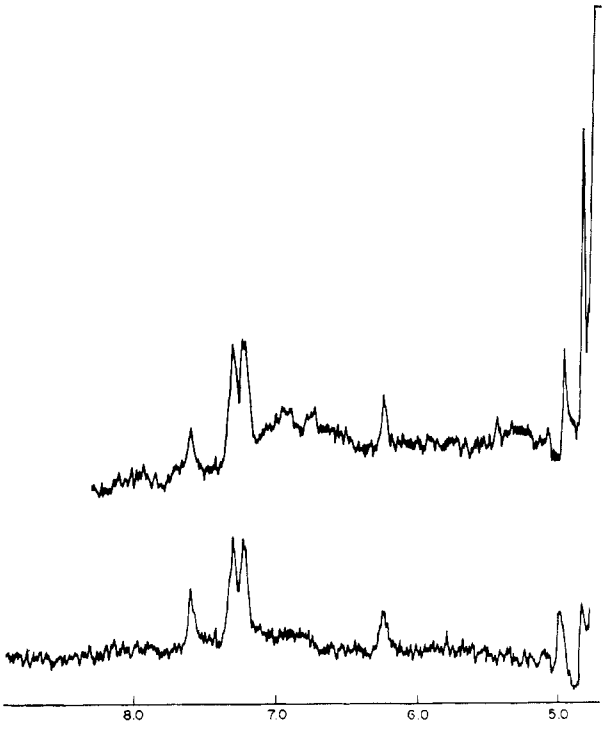

FIGURE 7: Aromatic region, [12-β-pyrazolylalanine]-S-peptide$_{3-14}$. Lower trace: in absence of S-protein. Upper trace: 0.1 equiv of S-protein was added.

The peptide and analogs at 2% concentration in D_2O solution yield relatively simple and sharp spectra, in which the signals of many of the side-chain protons can be identified. The identification was made on the basis of previous surveys of the chemical shifts of peptides, such as that of Roberts and Jardetzky (1970), and confirmed by examination of the spectra of synthetic subfragments of S-peptide, previously prepared in these laboratories (Hofmann *et al.*, 1965a,b,c). The S-protein at the lower concentrations gave broad spectra of low intensity, which did not interfere with the observation of the S-peptide spectra. At the same time exchange between the bound and free states resulted in changes in the peptide spectra which were readily visible. The changes were proportional to the amount of protein added, confirming their origin.

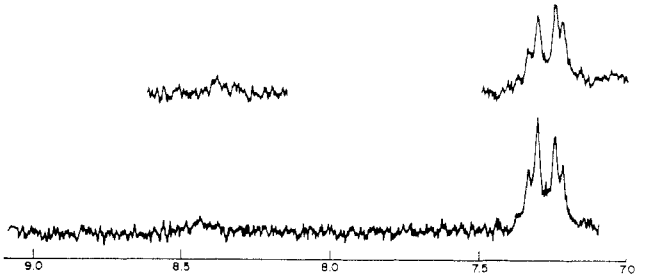

FIGURE 9: Aromatic region, [10-ornithine,12-(3-carboxymethylhistidine)]-S-peptide$_{1-14}$. Lower trace: in absence of S-protein. Upper trace: 0.1 equiv of S-protein was added.

Experimental Section

Spectra. Frequency swept nmr spectra were recorded using the MPC-HF spectrometer, which has been described elsewhere (Dadok *et al.* 1970). Proton spectra were recorded at probe temperature (31.5 ± 0.5°) at 250 MHz, using residual H_2O in the D_2O solutions as an internal lock. Reported shifts are relative to the water peaks, taken as 4.75 ppm downfield from Me_4Si. Usually 1500-Hz portions of the spectra upfield and downfield from water were recorded separately; sweep times of the order of 1500 sec and system response times of 0.3–1.0 sec were employed. Changes in intensity and width of lines were estimated visually after superposition of corresponding spectra on a light table.

Samples. Solutions were prepared by dissolving a weighed sample of the appropriate peptide in D_2O, lyophilizing, and redissolving in D_2O to yield 2% solutions. The solutions were not degassed. The pD was near 6.0. They were loaded into standard 5-mm precision nmr sample tubes. Aliquots of S-protein solution at ~ 20% in D_2O, prepared by the same technique, were added using a microliter pipet.

Materials. Chromatographically and analytically homogeneous samples of peptides 1–6, Table I, were prepared as described (Hofmann *et al.*, 1966b; Hofmann *et al.*, 1969; Hofmann *et al.*, 1966a; Hofmann *et al.*, 1971).

Results

The upfield portions of the spectra of S-peptide, free and in presence of 0.1 equiv of S-protein, are shown in Figure 1. The

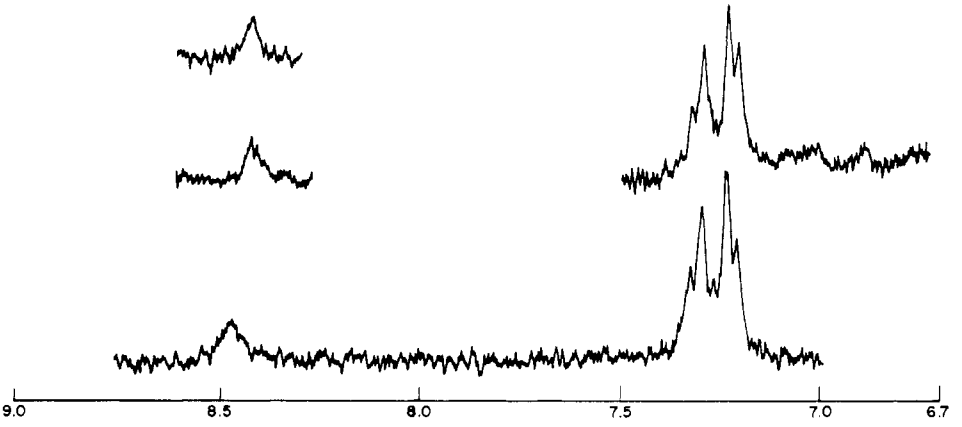

FIGURE 8: Aromatic region, [12-(3-carboxymethylhistidine)]-S-peptide$_{1-14}$. Lower trace: in absence of protein. Upper traces: 0.1 equiv of S-protein was added.

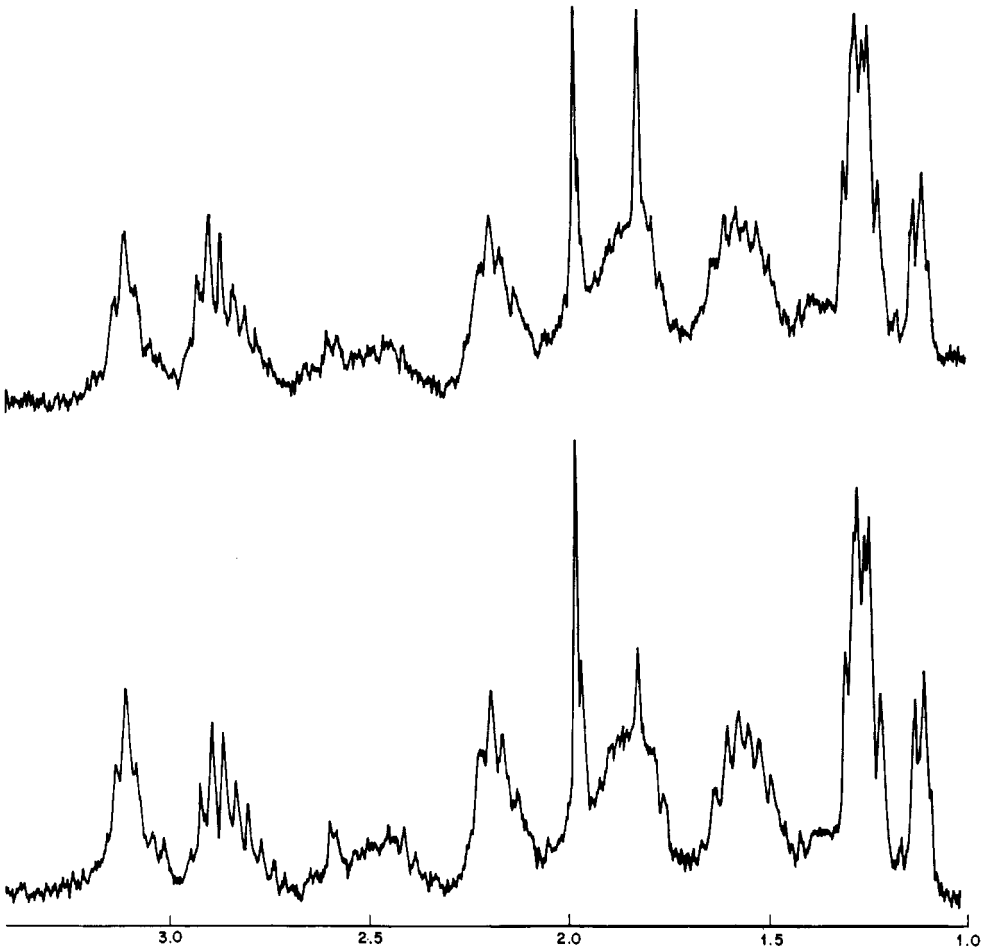

FIGURE 10: [12-(3-Carboxymethylhistidine)]-S-peptide$_{1-14}$, 2% in D_2O. Lower trace: in absence of protein. Upper trace: 0.1 equiv of S-protein was added.

most obvious changes occur in the resonances at δ 3.2 and 2.0. The former arises from the δ protons of the arginine side chain and the β protons of the phenylalanine; the latter sharp singlet, assigned to the SCH_3 of the methionine, becomes broader and less intense compared to the remaining peaks.

We have confirmed the observations of Meadows and Jardetzky (1968) that the histidine protons are not observable in the downfield portion of the spectrum, nor does addition of S-protein render them visible.

The upfield portions of the spectra of synthetic S-peptide$_{1-14}$ free and in the presence of 0.08 equiv of S-protein are shown in Figure 2. Changes similar to those observed with S-peptide are seen in this case also. The changes vary linearly with the amount of added S-protein. In addition the histidine protons are observable in the 1–14 peptide. Figure 3 shows the effect on the histidine proton signals of adding 0.1, 0.2, and 0.4 equiv of protein to the 1–14 peptide. There is a shift to higher field, proportional to the amount of added protein. Since the affinity constant is high, all the protein will be complexed in these mixtures. From the observed shifts the shift in the complex is calculated to be 0.25 ppm upfield.

A reasonable explanation may be offered for these observations. The methionine is known to be important for binding; van der Waals forces holding it against the valine-47 and leucine-51 side chains immobilize it sufficiently in the complex that dipolar broadening by the protons of the valine and leucine methyls becomes significant.

It has been suggested (Scatturin *et al.*, 1967) that S-peptide is in a random-coil conformation in solution and that the three turns of helix are formed concomitant with binding to S-protein. If this is so, the histidine-12 protons would be brought into position 5–6 Å in front of the plane of the benzene ring of phenylalanine-8 in the complex, but would not approach it closely in the free peptide. An upfield shift of about 0.25–0.5 ppm would be predicted for such an arrangement, in agreement with that observed.

Models suggest that hydrogen bond and salt-bridge formation can take place between the free carboxylate of glutamic acid-2 and the guanidinium group of arginine-10 in the complex; indeed the X-ray diffraction data indicate that arginine-10 is correctly disposed for this interaction. If the free peptide is in a random-coil arrangement, binding to the protein and the formation of this salt bridge would result in an immobilization of the arginine side chain and a broadening of the δ-proton signals, as is observed, so that this observation is also consistent with the suggestion of Scatturin *et al.* (1967).

In order to test some of these conclusions, the spectral behavior of the [10-β-pyrazolylalanine]-S-peptide$_{2-14}$ and [10-β-pyrazolylalanine]-S-peptide$_{3-14}$ in the presence and absence of S-protein was investigated. The above reported upfield shifts in the imidazole resonances of histidine-12 on binding might be ascribed to different protonation levels arising from pH or medium changes accompanying the addition of S-protein to peptide. The β-pyrazolylalanine analogs, however, differ in the pK_a of the base groups by about 3 units

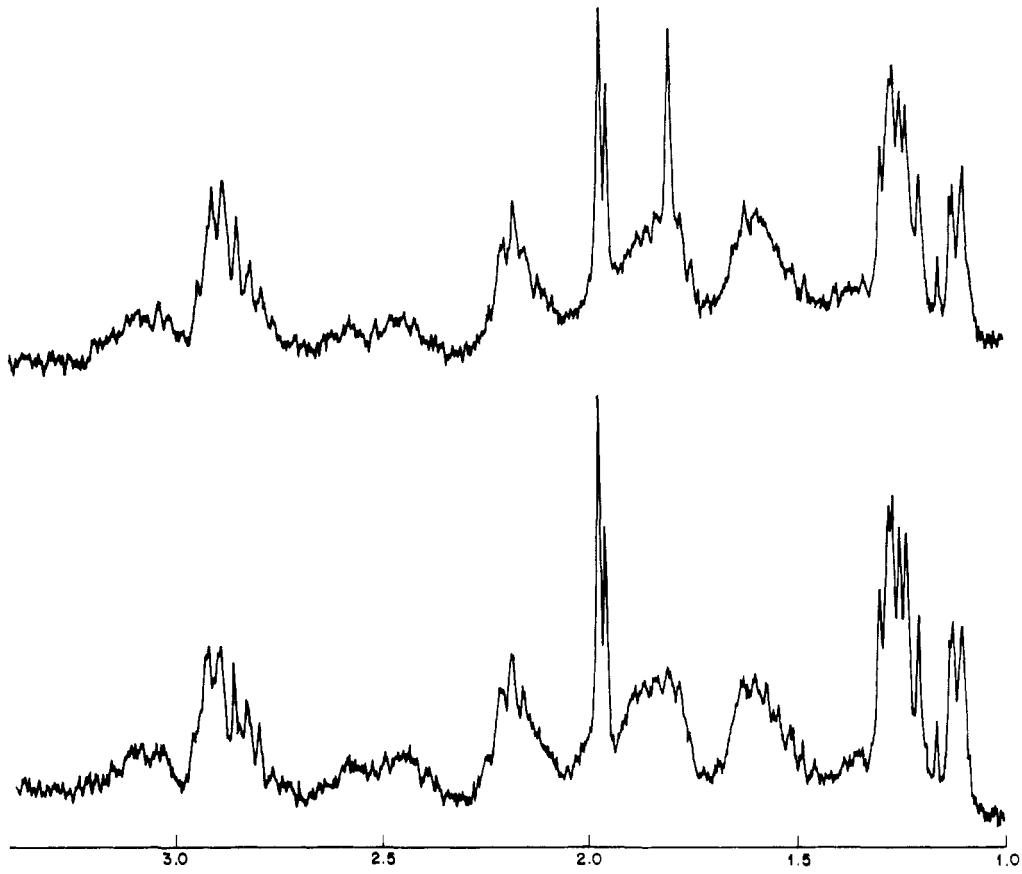

FIGURE 11: [10-Ornithine,12-(3-carboxymethylhistidine)]-S-peptide$_{1-14}$ 2% in D_2O. Lower trace: in absence of protein. Upper trace: 0.1 equiv of S-protein was added.

(Schneider and Schaeg, 1962), so that an upfield shift observed under the same circumstances would be very unlikely to come from deprotonation. In fact, [12-β-pyrazolylalanine]-S-peptide$_{2-14}$ yields the same spectral changes when S-protein is added; the β-pyrazolyl ring proton signals undergo an upfield shift, the arginine δ-proton signals are broadened, and the methionine SCH_3 proton peak is broadened and less intense (Figures 4 and 5). On the other hand, in [12-β-pyrazolylalanine]-S-peptide$_{3-14}$, these changes are not observed (Figures 6 and 7). The absence of glutamic acid-2, and the decrease in stability resulting from the loss of the glutamate-2–arginine-10 bridge, results in lowered binding (Hofmann *et al.*, 1971; Finn and Hofmann, 1965).

The importance of the contribution of arginine-10 to this interaction was investigated by comparing the spectra of [12-(3-carboxymethylhistidine)]-S-peptide$_{1-14}$ and [10-ornithine,-12-(3-carboxymethylhistidine)]-S-peptide$_{1-14}$. Carboxymethylation of histidine-12 on the 3 nitrogen has been shown to increase binding of peptide analogs while destroying the enzymatic activity of the complex. Both peptides were shown to be competitive inhibitors of S-peptide; however, substitution of the strongly basic guanidinium group of arginine-10 by the less basic amine group of ornithine with concomitant shortening of the side chain resulted in somewhat decreased binding, presumably because of the inability of ornithine-10 to interact with glutamic acid-2 (Hofmann *et al.*, 1970).

In the low-field region, only the signal from the 2 proton of the imidazole ring is observable, the 4-proton signal being obscured by the phenylalanine ring protons. It is somewhat broadened, presumably as a result of interaction with the 3 nitrogen which may undergo quadrupolar relaxation more slowly in the 3-carboxymethyl derivatives. The usual upfield shift is observed upon addition of protein (Figure 8). The resonance in the case of the [10-ornithine,12-(3-carboxymethylhistidine)] analog is very broad and the shift is not so clearly established in this case (Figure 9).

In [12-(3-carboxymethylhistidine)]-S-peptide$_{1-14}$, the arginine and methionine signals exhibit similar changes to those reported above (Figure 10). When arginine-10 is replaced with ornithine, the δ-proton signals of the side-chain shift upfield and are superimposed on those of the lysine ϵ protons (Figure 11). This complex of signals is, however, unchanged on addition of S-protein, so that the ornithine side chain appears not to be involved in the binding, as expected. The SCH_3 peak broadens and diminishes, signalling its binding. The sharp peak appearing immediately adjacent to the *S*-methyl peak is assigned to the CH_2 of the carboxymethyl group.

Conclusions

The binding of S-peptide and its analogs to S-protein is accompanied by an upfield shift of the histidine-12 ring proton signals and a broadening and diminution in intensity of the signals from the arginine-10 δ protons and the methionine SCH_3 protons. These observations are in accord with a model of binding which postulates a random-coil conformation for the free S-peptide and the formation of a salt-bridge link between glutamate-2 and arginine-10 on binding. The findings are consistent with the structures deduced for ribonuclease S by X-ray diffraction.

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A Magnetic Susceptibility Study of Hemerythrin Using an Ultrasensitive Magnetometer*

James W. Dawson, Harry B. Gray,† H. Eckhardt Hoenig, George R. Rossman, Jeffrey M. Schredder, and Run-Han Wang

ABSTRACT: The magnetic susceptibilities of oxy- and metaquohemerythrin in the range 3–200°K have been determined using an ultrasensitive superconducting quantum magnetometer, and for the first time their antiferromagnetic components have been conclusively resolved. The exchange coupling constants, J, between the two high-spin iron(III) atoms in each subunit are -77 and -134 cm^{-1}, respectively. The electronic spectra of these two proteins in deuterium oxide have been measured from 800 to 1200 nm but no additional bands were obtained. After comparison to magnetic and spectroscopic data obtained previously for ferric dimer model complexes, it is concluded that the two iron(III) atoms in subunits of both oxy- and metaquohemerythrin are oxo bridged.

Hemerythrin is the oxygen-carrying nonheme iron protein found in the invertebrate phyla: sipunculids, brachiopods, polychaetes, and priapulids. The nature of the iron in the oxy, deoxy, and met (oxidized) forms of this protein from the sipunculid *Golfingia gouldii* has been studied by physical methods such as Mössbauer spectroscopy (Okamura *et al.*, 1969; York and Bearden, 1970; Garbett *et al.*, 1971), electronic spectroscopy (Garbett *et al.*, 1969; Gray, 1971) and magnetic susceptibility determinations (G. Gunther, 1969, personal communication; S. Simon and G. R. Rossman, 1969, unpublished data; Okamura *et al.*, 1969; York and Bearden, 1970; Moss *et al.*, 1971; Gray, 1971).

The hemerythrin (mol wt 108,000) from *G. gouldii* consists of eight subunits, each of which contains two iron atoms capable of binding one oxygen molecule (Klotz and Keresztes-Nagy, 1963). As the molecular weight of each subunit is 13,500, conventional susceptibility determinations (Earnshaw, 1968; Figgis and Lewis, 1965) are of little value in determining the magnitude of any possible magnetic interactions between the two iron atoms. Even a superconducting

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† To whom correspondence should be addressed.