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Implication of Tyrosine in Iron Binding in Hemerythrin*

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ABSTRACT: In a study of the nitration of hemerythrin by tetranitromethane it has been found that in the apoprotein all five possible tyrosines react, but in oxyhemerythrin and in methemerythrin monomer only three residues react. Pepsin hydrolysis of nitrated oxyhemerythrin yields two yellow spots on peptide mapping. The slower moving spot contains 2 moles of nitrotyrosine arising from tyrosine 18 and 70. The faster moving spot is a peptide containing nitrotyrosine 67. The results suggest unreacted tyrosines 8 and 109 are each chelated to iron. There was no differential reactivity toward *N*-bromosuccinimide oxidation, suggesting tryptophan is not a ligand to iron.

We have recently shown that four of the seven histidine residues of the oxygen carrying nonheme iron protein, hemerythrin, serve as ligands to iron and may constitute a portion of the active site, Fan and York (1969). Assuming octahedral coordination and reserving a coordination position for oxygen which could be alternately occupied by water leave three ligands per iron atom unaccounted for. Besides the histidine just noted and the cysteine which has been ruled out, Keresztes-Nagy and Klotz (1965), the next most likely group to fill the remaining ligand positions is tyrosine.

The selectivity of tetranitromethane (TNM) for tyrosine and sulfhydryl groups under mild conditions has been demonstrated, Riordan *et al.* (1966, 1967). Also, it has been shown that iron protects coordinated tyrosine residues in transferrin against nitration by TNM, Line *et al.* (1967). These characteristics of TNM reactivity suggested to us that those residues of tyrosine in hemerythrin which were coordinated with iron would not be nitrated by TNM whereas those tyrosines not coordinated would react with TNM to form the yellow 3-nitrotyrosine. After pepsin hydrolysis those peptides containing the nitrotyrosine would be easily identifiable. Knowing specifically which residues did react would allow us to infer which were coordinated with the iron or buried and unavailable to the reagent. A preliminary account of this work has appeared, York and Fan (1970). It has been recently reported that TNM reacts with all five tyrosine residues in methemerythrin azide, pH 8, Rill and Klotz (1970). Under their experimental conditions these authors were not able to obtain any information as to the involvement of a specific tyrosine residue in the iron binding site although there was a qualitative implication of tyrosine as a ligand. Klippenstein *et al.* (1968) have published the amino acid sequence of hemerythrin which makes this study possible.

Materials and Methods

Preparation of Hemerythrin and Derivatives. Oxy- and methemerythrin were prepared as previously described, Fan and York (1969). Methemerythrin monomers were prepared by reaction with cyanogen bromide, Keresztes-Nagy and Klotz (1965). Heat-denatured hemerythrin which was soluble at pH 7–8 was prepared by heating a solution of oxyhemerythrin (2–10 mg per ml made up in 0.1 M phosphate at pH 8.0, 0.1% sodium lauryl sulfate, and 10^{-3} M in EDTA) at 50° until colorless. The solution was then dialyzed against the Tris–sodium lauryl sulfate buffer to remove the iron and EDTA.

Reaction with Tetranitromethane. The number of TNM reactive tyrosine residues in various forms of hemerythrin was determined in the following way. A working TNM (Aldrich) solution was prepared by saturating a 50% aqueous ethanol solution at room temperature. The concentration of TNM in this solution was determined by the amount of nitroformate, ϵ_{350} 14,000 M^{-1} cm^{-1}, Sokolovsky *et al.* (1969), released upon treating an aliquot with a large excess of cysteine at pH 7.0 in 0.1 M Tris chloride. At 27° this value was determined to be 25 μmoles of TNM/ml. All experiments were performed at a final concentration of 1 mg/ml of protein, 5–7% ethanol, and a 50-fold molar excess of TNM in 0.1 M Tris-chloride, pH 8.3. Complete reaction required 18 hr at 0°. After this time no further nitration of tyrosine in oxyhemerythrin occurred even with addition of fresh TNM. In order to remove nitroformate which interferes with the spectroscopic assay, the reaction mixture was dialyzed against 0.1 M Tris, pH 8.0, then precipitated with perchloric acid, redissolved in 0.1 M KOH, and dialyzed against 0.1 M Tris, pH 8.8, and the optical density was determined at 428 nm. The concentration of 3-nitrotyrosine was determined using ϵ_{428} 4100 M^{-1} cm^{-1}, Riordan *et al.* (1966). In order to study the kinetics, the reaction was stopped at a given time by the addition of excess cysteine which rapidly removed the excess TNM by oxidation of the cysteine,

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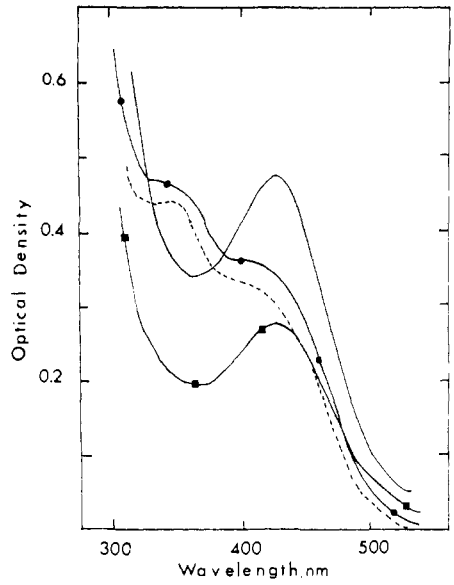

FIGURE 1: Spectra of the products of reaction of TNM with hemerythrin: (■) oxyhemerythrin and (—) apohemerythrin after acid precipitation, solubilization in 0.10 M base and pH adjusted to 8.8, (●) oxyhemerythrin after overnight dialysis, (- - -) oxyhemerythrin after extensive dialysis against 0.1 M Tris, pH 8.3. Protein concentration 0.32 mg/ml. Nitration was performed at 50-fold excess of TNM at pH 8.3 in 0.1 M Tris buffer for 16 hr at 0°.

TABLE I: Determination of the Number of Free Tyrosine Residues in Hemerythrin and Its Derivatives by Reaction with Tetranitromethane.[a]

Protein	Concn ($\times 10^5$ M)	OD_{428}	Calcd Concn[b] Tyr ($\times 10^5$)	Number Tyr Reacting
O_2 hemerythrin	3.4	0.42	10.28	3.00
Heat-denatured hemerythrin	2.0	0.47	11.00	5.10
Hemerythrin monomers	3.7	0.42	10.24	2.76
Methemerythrin	3.7	0.44	10.73	2.90
O_2 hemerythrin in 2 M urea	4.0	0.46	11.45	2.94

[a] Reaction performed in 0.1 M Tris chloride, pH 8.3 at 0° for 18 hr. [b] Calculated from ϵ_{428} of 4100, Sokolovsky *et al.* (1967), at pH 8.8 on hemerythrin derivatives which had been acid denatured, redissolved in base, and reprecipitated to remove interfering nitroformate.

Sokolovsky *et al.* (1969). The sample was then extensively dialyzed to remove by-products and the optical density was determined at 428 nm.

Isolation of Nitrotyrosine-Containing Peptides. For identification of the specific residues which were nitrated two procedures were employed. In the first procedure long peptides were obtained by a controlled pepsin hydrolysis of the heat-denatured nitrated hemerythrin. The heat-denatured material was suspended in 7 ml of 0.1 M KCl and the pH was adjusted to 1.9 on the Radiometer autotitrator. Pepsin was suspended in 0.1 M KCl and 0.5 mg was added to the hemerythrin suspension. The hydrolysis was performed at 37° at a constant pH of 1.9 until base uptake ceased. This procedure is referred to as the controlled hydrolysis.

In the second procedure short peptides were obtained by allowing extensive digestion of the hemerythrin by the pepsin. In this case the hemerythrin was acid denatured and was hydrolyzed by the pepsin overnight in formate buffer, pH 1.9, 0.1 M (formic acid–acetic acid–water, 55:156:1789). The pepsin to hemerythrin ratio was 1:20.

The products from both procedures were quickly evaporated to a small volume on a rotatory flash evaporator, the salt crystallized out, and the supernatant was spotted on Whatman No. 3MM paper. Electrophoresis was performed in the horizontal direction at 1700 V for 1.5 hr with formate buffer, pH 1.9. Chromatography was performed in the vertical direction using butanol–acetic acid–water (4:1:5) as the developing solvent.

Only two yellow spots appeared before ninhydrin spraying and these were near the origin. These spots were eluted and rechromatographed separately using the butanol–acetic acid–water solvent system. These two fractions were hydrolyzed in 6 N, triple-distilled HCl in a vacuum for 16 hr at 106°. Amino acid analysis was performed on the Beckman 120C analyzer.

Determination of Iron-Bound Tryptophan. The number of free tryptophan residues was determined by oxidation with *N*-bromosuccinimide (NBS) at pH 4.9. Hemerythrin solutions could be stabilized at pH 4.9 by dialyzing a hemerythrin solution initially at pH 8 against 0.1 M acetate, pH 4.9, containing 0.2% polyoxyethylene lauryl ether. Under these conditions, hemerythrin did not lose iron and the characteristic spectrum was maintained. Oxidation was performed in a cuvet thermostated to 0° by the addition of 5-fold molar excess of NBS in the acetate buffer containing 0.2% polyoxyethylene lauryl ether. The reaction was followed by the decrease in absorption at 280 nm and the concentration of reacting tryptophan was determined from the change in optical density according to Patchornik *et al.* (1958).

Miscellaneous Analytical Procedures. Hemerythrin concentration was determined from published extinction coefficients, Keresztes-Nagy and Klotz (1965). Denatured hemerythrin was determined from a microbiuret assay, Goa (1953). All spectra were recorded on a Cary 14 spectrometer. Iron was determined after wet acid ashing as the *o*-phenanthroline complex, Fortune and Mellon (1938).

Sedimentation coefficients were determined by centrifuging 0.2–0.4% hemerythrin solutions in 0.1 M Tris-chloride, pH 8.3, at 59,780 rpm in the Spinco Model E ultracentrifuge. Diffusion constants were determined in the ultracentrifuge at a speed of 4000 rpm in a synthetic boundary cell at a schlieren bar angle of 70° and calculated by the height-area method (Svedberg and Pedersen, 1940). Solvent used for the preparation of the synthetic boundary was the buffer against which the protein in question has been dialyzed.

Results

Nitration of Hemerythrin. The rate and extent of nitration of hemerythrin by tetranitromethane could not be followed by direct photometric assay because of overlap of the nitroformate and iron hemerythrin spectra with that of nitrotyrosine. Figure 1 shows the spectra of nitrohemerythrin after dialysis overnight *vs.* dialysis with frequent buffer changes in

TABLE II: Effect of Modification of Hemerythrin by Tetranitromethane and *N*-Bromosuccinimide on the Iron Content.

Protein	Reagent	Iron Content: Calculated (μmole)	Iron Content:- Observed after Reaction and Dialysis (μmole)	% Denaturations[a] Resulting from Modification
Oxyhemerythrin (4 mg)	TNM	0.59	0.58	1.7
Methemerythrin (4 mg) (SCN complex)	TNM	0.59	0.55	6.8
Oxyhemerythrin (3.2 mg)	NBS	0.47	0.44	6.4
Methemerythrin (5 mg) (SCN complex)	NBS	0.74	0.68	8.1

[a] Determined by iron loss after reaction and dialysis. Reaction conditions were those considered optimum as described under Methods.

comparison to nitrohemerythrin which had been repeatedly precipitated by perchloric acid and redissolved in 0.1 M sodium hydroxide, and the final pH was adjusted to 8.8 with Tris (0.1 M) buffer. Only after extensive dialysis (frequent buffer changes over a 12-hr period) did the optical density at 428 of the undenatured nitrohemerythrin approach the correct value as given by the denatured, washed material. The extent of nitration was therefore obtained from the optical density at 428 nm of the acid-denatured, washed material and from amino acid analysis. All forms of hemerythrin studied, with the exception of the heat-denatured hemerythrin, had three tyrosine residues nitrated under the conditions indicated (Table I). Five residues were nitrated in the heat-denatured hemerythrin.

The kinetics of the reaction was studied at 0° and 23° and at a 10- and 50-fold molar excess of TNM (Figure 2). With a 10-fold molar excess the rate of the reaction was initially the same as that with a 50-fold excess but then slowed; consequently, the maximum of three nitrated residues was not obtained. With a 50-fold molar excess the nitration was essentially complete in 16 hr at 0° and in 80 min at 23°. This latter value shows considerable variation depending upon the form of the protein, *i.e.*, oxy or met, and to some extent the age of the preparation.

Spectroscopic evidence suggested that three tyrosine residues were nitrated under the conditions described and with no concomitant loss of iron (Table II). To corroborate these results, amino acid analysis was performed on the hydrolysates of the nitrated hemerythrins. Table III shows a comparison of the amino acid composition of the untreated, the nitrated, and the nitrated, heat-denatured hemerythrin. These results indicate the presence of two free tyrosines and 3.2 nitrotyrosine residues in the nitrated hemerythrin and no free tyrosine, 3.5 mononitro-, and 1.3 dinitrotyrosine in the nitrated, heat-denatured hemerythrin.

TABLE III: Amino Acid Composition of Hemerythrin and Its Nitrated Derivatives.

	Residues per Subunit of 13,500		Nitrated Denatured Hemerythrin[a]
	Oxyhemerythrin	Nitrohemerythrin	
Lys	10.9	11.1	11.0
His	6.8	6.8	6.9
Arg	3.3	3.4	3.3
Asp	16.9	19.3	18.0
Thr	4.6	5.1	5.1
Ser	3.6	4.0	4.1
Glu	9.9	10.2	10.4
Pro	4.2	4.2	4.0
Gly	6.4	6.1	5.9
Ala	5.5	5.2	5.3
½ Cys	1.1	0.8	0.7
Val	3.9	4.3	3.9
Met	1.1	1.1	1.0
Ilu	8.7	8.9	9.0
Leu	7.7	7.9	8.1
Tyr	5.0	2.0	0.0
Phe	8.8	8.4	8.9
NO_2 Tyr	0.0	3.2	3.5
di-NO_2 Tyr	0.0	0.0	1.3
	108.3	112.0	110.4

[a] Prepared at 0°. Other conditions as described under Methods.

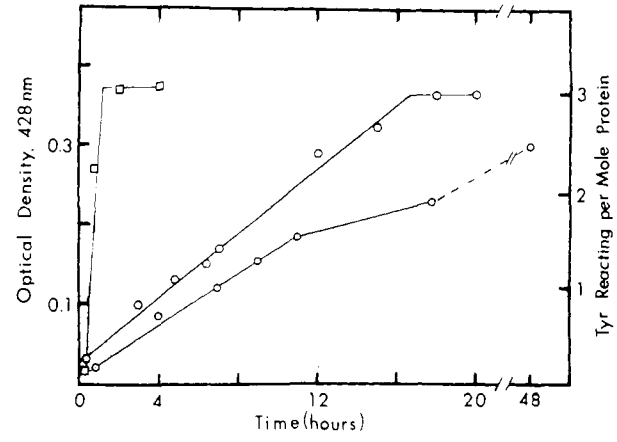

FIGURE 2: Kinetics of the nitration of oxyhemerythrin with tetranitromethane: (—□—) temperature 23°, 50-fold excess of TNM; temperature 0°; (—○—) 10-fold excess of TNM and (—○—) 50-fold excess of TNM. All experiments were performed at pH 8.3 in 0.1 M Tris-chloride. Kinetics were followed by addition of excess cysteine to an aliquot to remove excess TNM, followed by extensive dialysis and determination of the optical density at 428 nm.

FIGURE 3: Tracing of peptide map of the hydrolysate from the short controlled pepsin hydrolysis of nitrated oxyhemerythrin. Spots marked "Y" were yellow. The other peptides were detected by spraying the paper with 0.2% ninhydrin in acetone and heating. Details are described in the Materials and Methods section.

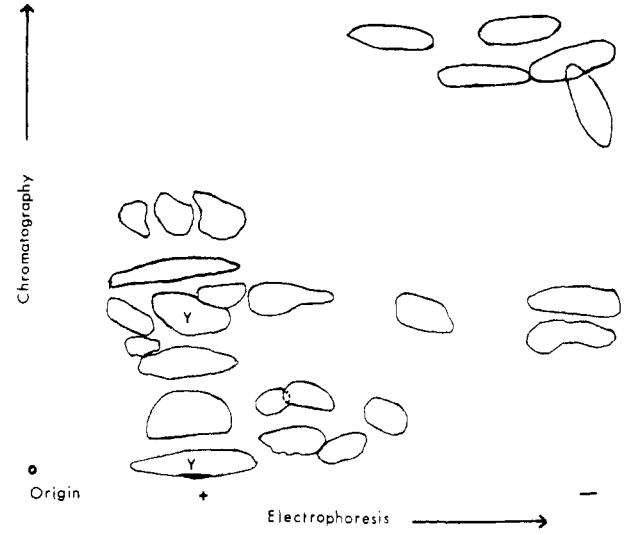

FIGURE 4: Tracing of peptide map of the hydrolysate from the extensive pepsin hydrolysis of nitrated oxyhemerythrin. Spots marked "Y" were yellow before ninhydrin spraying.

Nitrohemerythrin prepared from oxyhemerythrin at 0° and known to contain 3 nitro groups has an s_{20} of 1.9 and D_{20} of 6.2×10^{-7}. The value of f/f_{min} calculated from s is 1.70 and from D is 1.74. The molecular weight calculated from s and D is 28,100. The molecular weight determined from an equilibrium run on the Model E was 28,200. These values suggest a dimer; however, the protein moves on a Sephadex G-200 column more like a monomer than a dimer (Table IV).

Isolation of Nitrotyrosine Peptides. The nitrohemerythrin which had been heat denatured was rapidly hydrolyzed by pepsin at pH 1.9 and showed a base uptake equivalent to 20 bonds cleaved. In contrast, pepsin hydrolysis of acid-denatured material was exceedingly slow and required 16–18 hr for complete reaction. Figures 3 and 4 show the peptide maps of the pepsin hydrolysates of the heat- and acid-denatured nitrohemerythrin, respectively. The spots marked "Y" are the yellow spots which were visible before ninhydrin spraying of the paper and therefore contain the nitrotyrosine peptides. The spot nearest the origin proved to be free nitrotyrosine in both preparations. In the case of the heat-denatured material, the second yellow spot (peptide A) had the amino acid composition shown in Table V. In the more extensively hydrolyzed acid-denatured nitrohemerythrin, the second spot (peptide B) moved faster on electrophoresis as well as chromatography and upon hydrolysis and amino acid analysis was shown to contain only glutamic acid and nitrotyrosine (Table VI). The peaks which we have identified

TABLE IV: Characterization of the Product of TNM Nitration of Oxyhemerythrin on Sephadex G-200.[a]

Protein	Mol Wt	$D_{20} \times 10^7$	V_e (ml)	V_e/V_0
α-Chymotrypsinogen	23,200[b]	9.5[e]	94	2.30
Cytochrome *c*	12,400[c]	13.0[f]	110	2.69
Hemerythrin monomer	13,500[d]	12.3[d]	108	2.65
Nitrohemerythrin		6.2	103	2.53

[a] Oxyhemerythrin was nitrated at 0° as described under Methods. This material was then passed through a 29 × 2.2 cm Sephadex G-200 column previously equilibrated in 0.05 M Tris chloride, pH 8.3, V_0 = 41 ml as determined with Dextran Blue-2000. [b] Schwert (1951). [c] Margoliash (1962). [d] Klotz and Keresztes-Nagy (1963). [e] Wilcox *et al.* (1957). [f] Edsall (1954).

TABLE V: Amino Acid Composition of Nitrotyrosine Containing Peptide A.[a]

Amino Acid	Observed				Expected for Peptide 56–67
Asp	2.6	1.6	1.6	1.7	1
Thr	1.0	0.7	0.6	0.8	0
Ser	1.3	0.9	0.9	0.9	1
Glu	2.1	2.9	3.5	3.0	4
Ala	1.1	2.0	1.0	1.0	1
Val	1.2	1.2	0.9	0.9	1
Met	1.0	1.0	1.0	1.0	1
Ile	1.6	1.5	1.1	1.1	0
Leu	1.9	1.7	1.7	1.6	2
NO_2-Tyr[b]	2.1	3.0	1.5	1.6	1
NO_2-Tyr from Tyr 70 and 18		0.0	1.6	1.5	2
Total NO_2-Tyr		3.0	3.1	3.1	3

[a] Peptide A was obtained from a short controlled pepsin hydrolysis of nitrohemerythrin. [b] High values are due to contamination of nitrotyrosine from the free nitrotyrosine arising from tyrosines 18 and 70. This free nitrotyrosine was difficult to separate from the peptide 56–70 on a preparative scale.

TABLE VI: Amino Acid Composition of the Two Yellow Peptides from Extensive Pepsin Hydrolysis of Nitrated Hemerythrin.

Spot on Peptide Map	Amino Acid	Observed		Peptide Yield (%)
Faster moving yellow spot	Glu	1.1	1.1	40
	NO_2-Tyr	1.0	1.0	
Slower moving yellow spot	NO_2-Tyr	2.1	1.7	68 and 84

as nitrotyrosine and dinitrotyrosine have the same retention time on the long column of the analyzer as reported previously, Sokolovsky *et al.* (1967). The nitrotyrosine was obtained in 70–80% yield, peptide A in 50% yield, and peptide B in 40% yield.

Oxidation of Tryptophan. Oxidation of tryptophan by *N*-bromosuccinimide did not cause the release of any iron from the protein (Table II). In both the iron protein and apoprotein 3.5–4.0 moles of tryptophan were modified without loss of iron.

The ultraviolet spectrum was not lost as a result of tryptophan oxidation (Figure 5). The small decrease in extinction of the NBS-treated material shown in Figure 5 is due to dilution by added NBS plus a small amount of denaturation. This spectrum was taken after 4 tryptophan residues were oxidized as determined by diluting an aliquot of the reaction mixture on which this spectrum was taken and determining the change in absorbance at 280 nm (Table VII).

During the pH adjustments to 4.9 at 4° by dialysis there is a 20% loss of hemerythrin by denaturation if polyoxyethylene lauryl ether is not included. Any insoluble protein is removed and free iron is dialyzed out. The success of the NBS oxidation and retention of the characteristic hemerythrin spectrum can be contributed to the use of the polyoxyethylene lauryl ether in the reaction medium. Sodium lauryl sulfate was not effective in preventing denaturation.

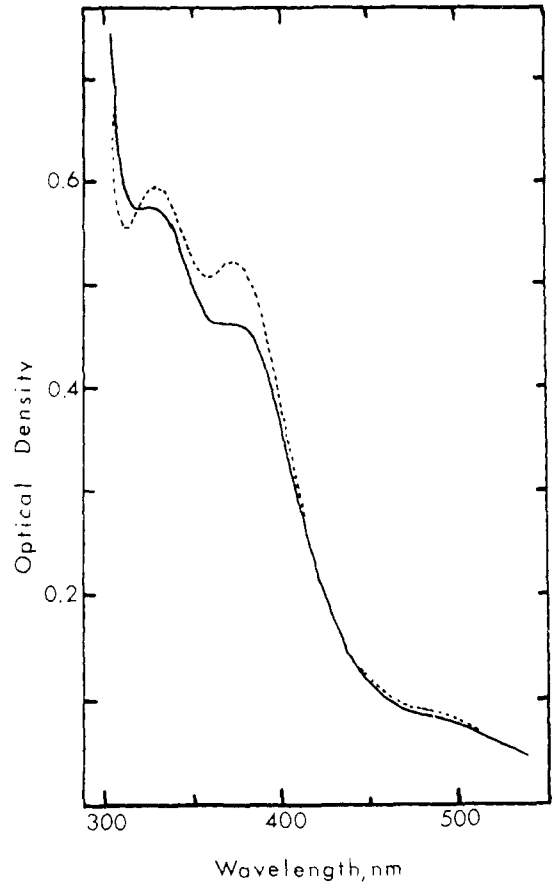

FIGURE 5: The effect of *N*-bromosuccinimide oxidation on the spectra of hemerythrin acetate: (– – –) before and (———) after addition of 5-fold excess NBS in pH 4.9, 0.1 M acetate buffer containing 0.2% polyoxyethylene lauryl ether. Oxidation of 4 moles of tryptophan was verified by decrease in 280-nm absorption. The NBS excess is expressed in terms of moles of NBS to moles of hemerythrin.

Discussion

In any study which attempts to determine by differential chemical reactivity the amino acid residues which constitute the active site of a metalloprotein, one is faced with three interpretations of the results. In the metalloprotein the residues which do not react could be those which are bound to the iron, those which are at the subunit binding site, or those which are inaccessible to the reagent. In addition one must be able to demonstrate that no alteration of the active site occurred as a consequence of the modification. Maintenance of the spectrum attributed to the active site during the course of the modification would constitute unequivocal proof of the "nativeness" of the modified protein. Distinguishing between lack of reactivity of a particular residue because of chelation to the metal or because of its being hidden in the protein matrix is very difficult. From the data presented here, lack of reactivity of the two tyrosine residues could be attributed to either cause; however, we prefer to believe the nonreactivity is a result of chelation to iron. Work is in progress to prepare a native apoprotein. A study of its reactivity with TNM would allow us to distinguish between the two possible interpretations of the present data.

Lack of reactivity because of involvement in subunit binding can be ruled out. Table I shows that it is immaterial whether hemerythrin polymer or monomer is reacted with

TABLE VII: Determination of Free Tryptophan Residues in Hemerythrin by Reaction with NBS.

Protein	Protein Concn (10^5 M)	Δ OD_{280}	Calcd Trp Concn (10^5 M)	No. of Trp per Subunit
Methemerythrin (acetate complex)[a]	2.52	0.36	9.43	3.8
Methemerythrin (thiocyanate complex)[b]	2.44	0.32	8.38	3.5
Nitrated hemerythrin[c]	1.80	0.25	6.51	3.6
Apohemerythrin[d]	1.90	0.29	7.60	4.0

[a] Reaction performed in 0.02 N acetate, pH 5. [b] Reaction performed in 0.1 N acetate, pH 4.9, plus 0.2% polyoxyethylene lauryl ether. [c] Reaction performed in 0.1 N acetate–formate, pH 3.2. [d] Heat denatured. Reaction conditions *b*.

TNM; only three tyrosine residues react in either case. After acid denaturation the two remaining residues are nitrated.

Proof of the presence of the active site of hemerythrin after reaction with TNM is equivocal. The appearance of the broad 428-nm absorption of the 3-nitrotyrosyl residues makes the detection of the anionic ligand "charge transfer" bands of hemerythrin (Keresztes-Nagy and Klotz, 1965), which occur in this region, difficult. The spectrum shown in Figure 1 with an absorption in the 350- to 360-nm region is very similar to that of Rill and Klotz (1970) for their "extensively degraded" nitrohemerythrin. We have found that this absorption arises from protein-bound nitroformate which is a product of the nitration (λ_{max} 350 nm). This conclusion is based on the observation that after extensive dialysis of nitrated oxyhemerythrin, to the point where no nitroformate can be detected spectrally in the dialysate, nitroformate is released into the supernatant on acid denaturation of the protein. This protein after redissolving in base and adjusting the pH to 9 shows a loss of the 350- to 360-nm absorption and a more clearly defined spectrum of the nitrotyrosyl protein (Figure 1). The 330-nm absorption characteristic of the active site of hemerythrin is not observed against the background of nitrotyrosine and protein-bound nitroformate. Gray (1970) has presented evidence that suggests the 330-nm absorption arises from the antiferromagnetically coupled pair of iron atoms present in oxy- and methemerythrin, Garbett *et al.* (1969); York and Bearden (1970). Therefore, the loss of the 330-nm peak indicates a breakdown of the iron–iron coupling and does not suggest any change in the ligands binding the individual iron atoms. Although iron is not lost on the modification of three tyrosine residues (Table II), and assuming the two tyrosines do not react because they are chelated to iron, it is not possible to unequivocally assign a tyrosine–iron chelate structure to the native active center of hemerythrin based on the present data. The tyrosine–iron chelate suggested by this study could arise from ligand exchange if alteration of the active center occurs on modification.

The nitrated hemerythrin was analyzed in an attempt to ascertain its nativeness. Nitration does lead to a large change in the frictional coefficient (1.13 to 1.70) and a decrease in the diffusion constant. The molecular weight calculated from s and D and from sedimentation equilibrium is 28,000 which is consistent with a dimer; however, the material behaves on a Sephadex G-200 column as a monomer (Table V). If the material were moving as a dimer on Sephadex then it would be expected to elute near chymotrypsinogen since the diffusion constant is smaller and the molecular weight is slightly larger than that of chymotrypsinogen. The dimer seen in the ultracentrifuge is not a covalently linked dimer arising from cross-linking of tyrosyl radicals generated by TNM as has been reported in trypsin and trypsinogen, Holeysovsky *et al.* (1969), and in the cytochromes, Skov *et al.* (1969), because all the tyrosine can be accounted for as tyrosine or nitrotyrosine (Table III). In the cases cited where cross-linking occurs, there is a loss of tyrosine.

In contrast to other reports of significant alteration of tryptophan by TNM, Cuatrecases *et al.* (1968) and Muhlrad *et al.* (1968), under the conditions of these experiments no more than 0.2 mole out of 3.6 moles could be attributed to alteration (Table VII) of tryptophan by TNM.

Limited pepsin hydrolysis of the nitrated iron protein yields a peptide whose composition suggests that it comprises residues 56 through 67. In the chromatographic system used, the apparently contaminating threonine and isoleucine could not be removed. The peptide was also high in aspartic but low in glutamic acid, assuming the sequence of Klippenstein (1968) is correct. The sequence of this nitrotyrosine-containing peptide was not determined; however, from the composition and the fact that it contains the only methionine residue, it is certain that Tyr-67 is one of the nitrated residues. The peptide is reasonable from the viewpoint of pepsin specificity with cleavage at Phe-54 and NO_2-Tyr-67.

The other spot isolated from this hydrolysis contained only nitrotyrosine which we assign as arising from residues 18 and 70, since both are coupled on their amino side by phenylalanine. From the specificity of pepsin one would expect cleavage on the carboxy side of NO_2-Tyr and carboxy side of Phe to yield 2 moles of free NO_2-Tyr. It is clear from these results that nitration of the Tyr does not inhibit the pepsin activity at the Tyr locus.

Since the peptide containing Tyr-67 was difficult to purify, a more complete pepsin hydrolysis was performed on acid-denatured material with the intent of isolating a shorter, more easily purifiable peptide containing Tyr-67. Again two yellow spots were obtained on mapping, one of which contained 2 moles of Tyr-NO_2 and the other 1 mole of pure dipeptide containing only Glu and Tyr-NO_2. This nitrotyrosyl peptide is different from the one obtained in the controlled hydrolysis (Figures 3 and 4). Analysis of the hemerythrin amino acid sequence shows that the combination of Glu and Tyr occurs at only one place in the sequence and that is at the site of Glu-66 and Tyr-67. The data from both peptides are in agreement that Tyr-67 is nitrated. This peptide has also been isolated as number C15 from chymotryptic digestion of hemerythrin, Subramanian *et al.* (1968).

The data indicate that the tyrosine residues which are nitrated in the oxy- or methemerythrin are residues 18, 67, and 70. This leaves residues 8 and 109 free to serve as ligands to the iron. Since Mossbauer data indicate that the chemical environment around each iron is identical (York and Bearden, 1970), we conclude that one tyrosine residue is bound to one iron atom.

These results were qualitatively confirmed by oxidation of nitrated hemerythrin with NBS followed by cleavage of the peptides and chromatography on a Bio-Rad P-2 column. One yellow peptide was obtained which ran with the solvent front. Smaller peptides were obtained which contained no nitrotyrosine. Since tryptophan occurs at positions 11 and 87, and nowhere in between, one large peptide should be obtained containing the three interior tyrosines which we have found to be nitrated. This finding corroborates the evidence from pepsin hydrolysis.

It is obvious from the data presented that tryptophan does not serve as a ligand to iron. All tryptophans are oxidized with no effect on the hemerythrin spectrum and no loss of iron.

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Topography of Nucleic Acid Helices in Solutions. Proton Magnetic Resonance Studies of the Interaction Specificities of Steroidal Amines with Nucleic Acid Systems*

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ABSTRACT: The interactions of steroidal amines with nucleic acid helices are reported. The studies were based on T_m of helix–coil transitions of nucleic acid–steroid complexes, as well as viscometric, circular dichroism, and temperature-dependent proton magnetic resonance techniques. The results indicate that the steroidal amines selectively stabilize the DNA helical structures, while causing the ribose-containing acids to unravel and denature.

The temperature-dependent proton magnetic resonance experiments show that single stranded random coils interact with the steroidal amines *via* hydrogen- and hydrophobic-type bonding. The capacity to form hydrogen bonding in the random coils is shown to be greater than that of the helical structure.

For the past 3 years, considerable work has been devoted in our laboratory to the elucidation of the interaction specificity of nucleic acid systems with mono- and polyammonium salts (Gabbay, 1967–1969; Glaser and Gabbay, 1968; Gabbay and Shimshak, 1968; Gabbay *et al.*, 1969a,b; Glaser and Gabbay, 1970; Passero *et al.*, 1970). The studies involved the use of diammonium salts of the general structure, I, $R_1R_2R_3N^+$-$(CH_2)_nN^+R_1R_2R_3 \cdot 2Br^-$. This paper reports the interactions of steroidal amines with nucleic acid helices. The effect of primary, tertiary, and quaternary steroidal ammonium salts as well as various epimers, *i.e.*, 3α-, 3β-, 17α-, and 17β-amino-5α-androstane, on the T_m of the helix–coil transition of deoxyhomopolymers, hybrid homopolymers, and ribohomopolymers are reported. Moreover, temperature-dependent studies of the proton magnetic resonance spectra of steroidal amines–nucleic acid complexes are utilized, together with ultraviolet absorption and circular dichroism studies, to elucidate the specificity of nucleic acid interactions.

Results and Discussion

T_m Studies with Steroidal Amines. Chart I lists the structure of the steroidal amines, II and III, used in this study. The synthesis and characterization of the salts II and III are reported

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