# THE ROLE OF TYROSINE IN THE HEMERYTHRIN ACTIVE SITE

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## SUMMARY

Results presented here show that all lysine and three of the five tyrosine residues can be modified in hemerythrin without any alteration in the spectrum characteristic of the iron binding site. The two remaining tyrosines become available to modification when the iron is removed suggesting they are chelated to iron. Modification of the three unchelated tyrosines results in dissociation of the octomer into monomers suggesting a role of these tyrosines in the subunit binding.

In a recent report (Fan and York, 1971) we presented evidence indicating that tyrosines 18, 70 and 67 reacted quickly with tetranitromethane and were not ligands to iron at the active site of hemerythrin, an oxygen transport protein in the sipunculids and brachiopods. The remaining two tyrosines, 8 and 109, did not react with tetranitromethane in the native protein but did react in iron free protein; consequently, it was suggested that the latter two tyrosines were either ligands to iron or were inaccessible to the reagent. In these studies we could not unequivocally determine if the active site was modified by the tetranitromethane because the absorption spectrum of the nitrated tyrosine residues overlapped that of the much less strongly absorbing active center of the hemerythrin. To circumvent this problem

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we have utilized N-acetyl imidazole to modify the free tyrosine residues. Since no new chromophores are produced by this reagent the spectrum of the hemerythrin active site can be followed as a function of tyrosine modification.

For these experiments the eleven lysine residues were first modified so they would not compete with the tyrosine for the acetyl imidazole then the amidinated protein was reacted with acetyl imidazole to modify the free tyrosine residues. This approach would allow an evaluation of the role of tyrosine in subunit dissociation phenomena independent of dissociation arising from modification of unprotected lysine by acetyl imidazole.

## METHODS

Hemerythrin used in these experiments was crystalline oxygenated material prepared as previously described (Florkin, 1933 and Klotz, 1957) and having an iron content of 0.81% and a $\epsilon_M$ of 3400 cm-1-mole$^{-1}$ per iron at 330 mu.

The lysine residues were amidinated with ethyl acetimidate hydrochloride prepared according to McElvain and Nelson, 1942. A fresh working solution of ethyl acetimidate was prepared by dissolving the solid in water and neutralizing with 2N NaOH to pH 9.0. The protein was amidinated by adding the ethyl acetimidate at 2 hour intervals to a solution initially 3% in oxyhemerythrin buffered to pH 9 in 0.1 N borate. Generally four additions of a 100 fold molar excess acetimidate to protein was required to achieve 95-100% conversion of lysine. The details were analogous to those of Wolfsy and Singer (1965). All reactions were performed at 0°. Excess reagent and hydrolysis products were remoded by dialysis against 0.1 M Tris-chloride, pH 8.3. The number of free lysine residues remaining after each addition of acetimidate were assayed by the trinitrobenzene sulfonic acid method previously described (Fan and York, 1969).

Amidinated oxyhemerythrin was acetylated with dry, crystalline N-acetyl imidazole (Eastman). The acetylation was performed at 0° on a 2% solution of amidinated hemerythrin at pH 7.5, 0.05 M in sodium borate and Tris-chloride. A 200 fold molar excess of acetylimidazole to hemerythrin tyrosine was added at 2 hour intervals. In general four additions were required for the number of tyrosines reacting to remain constant. Acetylimidazole and its hydrolysis products were removed by dialysis against the pH 7.5 mixed buffer before evaluation of the extent of O-acetylation was attempted. The number of unmodified tyrosine residues were evaluated by their reaction with tetranitromethane by measuring the nitrotyrosine chromophore absorption at 428 nm under conditions previously described (York and Fan, 1971) with the exception that the nitration was performed at pH 7.5 to prevent base catalyzed deacylation of the acetylated hemerythrin. After the nitration was complete the excess tetranitromethane was dialyzed out at pH 7.5 and 0° then the pH was adjusted to 8.8 for the spectral determination of the amount of nitrotyrosine formed. The amount

of O-acetylation was independently checked by the decrease of 278 nm absorption upon formation of the O-acetyl tyrosine. The absorbance was determined after dialyzing out excess reagents and their by-products. The $\Delta\epsilon_M$ was taken as 1160 (Riordan, Wacker and Vallee, 1965).

The percent denaturation of hemerythrin was determined after each modification by measuring the amount of iron which became soluble divided by the iron known to be protein bound originally. No attempt was made to remove soluble denatured material. Recoveries of 96 and 86 percent for amidinated and acetylated amidinated material was found. Sedimentation coefficients were determined by centrifuging 0.2 to 0.4% hemerythrin solutions in 0.1 M Tris-chloride, pH 8.3 in a double sector cell at 59,780 rpm in the Spinco Model E ultracentrifuge

## RESULTS AND DISCUSSION

All the lysine residues can be modified by amidination without changing

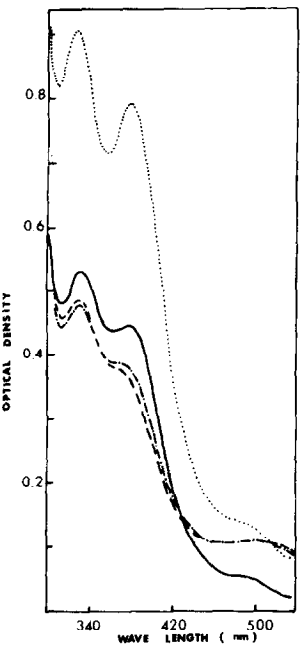


Figure 1. Absorption spectra of oxyhemerythrin (-----) 1 mg/ml, O-acetylated amidinated hemerythrin (-·-·-·-) 1 mg/ml, O-acetylated amidinated hemerythrin (·····) 1.95 mg/ml, and tetranitromethane treated O-acetylated amidinated hemerythrin (———) 1.05 mg/ml. Spectra of oxy- and amidinated oxyhemrythrin were taken in 0.1 M Tris-chloride, pH 8.3. Spectra of the acetylated samples were taken in 0.05 M Tris-borate pH 7.5.

the spectrum that is characteristic of the iron binding site. Figure 1 shows a typical oxygenated hemerythrin spectrum before and after amidination. Within experimental error the spectra are identical. Amidination was successful to the extent of 95%. Only 5% of the original TNBS sensi-

tive lysine residues remained to react with the trinitrobenzene sulfonic acid. We found that conversion of the $\epsilon$ amino group of lysine to an amidine renders it unreactive to trinitrobenzene sulfonic acid.

It is important to note that oxyhemerythrin maintained its oxygenated state after amidination as deduced from the spectrum, Fig. 1. In contrast to succinylation (Klotz and Keresztes-Nagy, 1963), where charge on the protein is altered and subunit dissociation occurs, amidination does not result in a change in total charge nor in subunit dissociation since the material sedimented as a single peak with a $S_{25,w}$ of 7.0. We feel this value is not significantly different from the 6.75 reported for native octomer (Klotz and Keresztes-Nagy, 1963). These findings strongly substantiate our earlier contention that the lysine residues and the amino group are not involved in the iron binding site as ligands and we now conclude they are not involved directly in the subunit binding site.

Tyrosine Modification: Acetylation of the available tyrosine residues in amidinated oxyhemerythrin with N-acetyl imidazole results in the oxidation of oxyhemerythrin and the formation of what spectrally appears to be methemerythrin chloride (Keresztes-Nagy and Klotz, 1965), Fig. 1. The extent of the tyrosine modification was assayed by reaction with tetranitromethane. As can be seen from Figure 1 no new peak at 428 nm characteristic of nitrotyrosine absorption appeared suggesting that no unchelated or unmodified tyrosine residues were available. Independently it was found that three ($3.1 \pm .2$) tyrosine residues were acetylated as measured by the decrease of 278 nm absorption. It was found that N-acetyl tyrosine ethyl ester (Sigma) would not undergo nitration by tetranitromethane under conditions identical to that used in the assay for acetyl amidinated hemerythrin. Denaturation of O-acetylated amidinated hemerythrin by heating at $80^{\circ}$ at pH 7.5 in 0.2% SDS Tris-borate buffer until the characteristic

hemerythrin spectrum disappeared resulted in the appearance of 2.1 to 2.4 residues of tyrosine which were tetranitromethane reactive. The model O-acetyl tyrosine ethyl ester was not deacylated by this procedure. The appearance of these two new tyrosines therefore represent the two iron ligands in hemerythrin which we had predicted earlier (York and Fan, 1971). The question of the possibility that these two tyrosines are buried residues is not experimentally approachable by these present techniques because procedures which unfold the protein and render these hypothetical groups available also cause the loss of iron and the release of potential ligands.

These results conclusively show that only three tyrosine residues can be O-acetylated in hemerythrin and that this modification does not affect the absorption spectrum characteristic of the active site; however, modification of these three tyrosine groups does cause dissociation of the octomer into the monomeric subunits which have a characteristic S value of 1.96 (Klotz and Keresztes-Nagy, 1963). Work is in progress to evaluate which one(s) of the three tyrosines is (are) involved in the subunit binding.

## ACKNOWLEDGMENT

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