

## Structural and Electrostatic Control of Chemical Modification of Histidine 64 Imidazole in Myoglobin with Cyanogen Bromide and Azide Ion

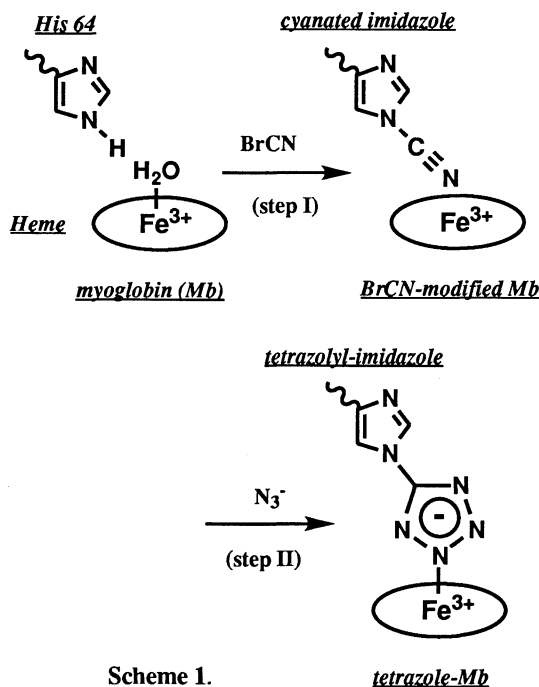
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I studied modification of the imidazole group of histidine (His) 64 in human myoglobin (Mb) and its mutant (Lys45→Arg; Mb(K45R)) with cyanogen bromide (BrCN) and azide ion ( $N_3^-$ ). In wild-type Mb, the modified protein was not obtained, while the modification was successful for mutant Mb(K45R), possibly due to an electrostatic stabilization of the -CN moiety with the Arg45 guanidyl group. This study implies that combination of chemical and recombinant DNA methods open a new method in the protein modification, where an unnatural group can be introduced into a specific site.

Recombinant DNA and chemical methods have been useful to make a mutant protein to delineate the functional importance of key residues in the biological activity of the protein. His64, so-called the distal histidine, is one of the target residues for the mutation to understand the ligand binding properties of Mb, i.e.  $O_2$ -storage hemoprotein. We have recently succeeded in specifically and chemically modifying the imidazole group of His64 of sperm whale Mb with BrCN and  $N_3^-$ ,<sup>1-3</sup> as shown in Scheme 1, and found that the modified-protein shows unique properties, compared with the native protein.<sup>3</sup>



Scheme 1.

This modification is very specific, so that the other part of the protein can be hardly modified, as was confirmed by the x-ray crystallography.<sup>2</sup> In this reaction, the step I is an electrophilic substitution of the aromatic imidazole ring with  $CN^+$ , possibly catalyzed by the iron, while the step II is a typical 1,3-dipole cycloaddition of  $N_3^-$  to the -CN moiety to yield the tetrazolyl-imidazole.

We have applied this modification to several Mbs from different sources, such as sperm whale (SW), horse heart (HH) and bovine heart (BH) Mbs. However, the modified protein was obtained only from SWMb, but not from others. This finding suggested that structural difference of SWMb from other Mbs are responsible for the modification of the His64 imidazole with BrCN and  $N_3^-$ . Comparing the primary structure among the Mbs, one of the most significant difference influencing properties of the heme and the His64 imidazole is likely the residue at the 45 position; Arg for SWMb, while Lys for others. The side chains of these groups, i.e. guanidyl group of Arg and  $\epsilon$ -amino group of Lys, lies between His64 imidazole and external solvent ( $H_2O$ ), so that they sterically and/or electrostatically interact with the His64 imidazole group. To test our idea that the residue at the 45 position plays a crucial role in the BrCN/ $N_3^-$  modification reaction, we examined here the BrCN/ $N_3^-$  modification of human Mb (HuMb) and its mutant, in which Lys45 in the wild-type protein was replaced with Arg (HuMb(K45R)) by the recombinant DNA method.<sup>4</sup>

Upon adding BrCN to the solution of wild-type HuMb, its optical absorption spectrum dramatically changed; the Soret absorption decreased in its intensity and shifted toward short

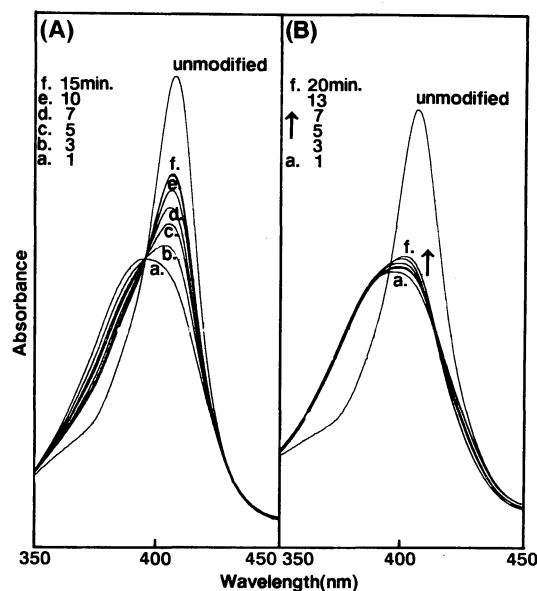
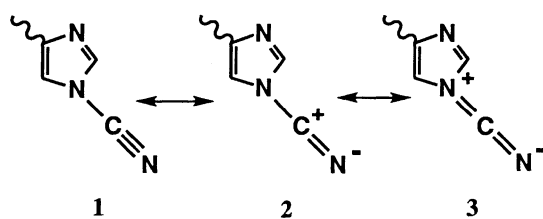


Figure 1. Absorption spectral changes of (A) wild-type human Mb(K45) and (B) its mutant Mb(K45R) upon addition of BrCN at room temperature in 0.1M phosphate buffer, pH 7.0. The mutant Mb(K45R) was kindly given from Prof. Steven G. Boxer and his co-workers (Stanford Univ, USA).<sup>4</sup> The spectra were recorded at the times (shown in the figure) after the BrCN addition.

wavelength (Figure 1A). The resultant spectrum showed characteristics of Mb with the cyanated-imidazole of His64.<sup>1</sup> However, as shown in Figure 1A, the spectrum rapidly changed, and was finally restored to that of the unmodified Mb, showing that the cyanated-imidazole is not stabilized in wild-type HuMb. In the next experiment, where  $N_3^-$  was added immediately after the BrCN addition, we obtained  $MbN_3^-$ , but not tetrazole-Mb. (I checked the formation of  $MbN_3^-$  or tetrazole-Mb in the following criterion;  $MbN_3^-$  is restored to its aquomet form after removing excess amount of  $N_3^-$  with gel-filtration, while tetrazole-Mb is not.) This result indicates that the modification in the Scheme 1 was unsuccessful for wild-type Mb. These observations were the same as the case for HHMb and BHMb.

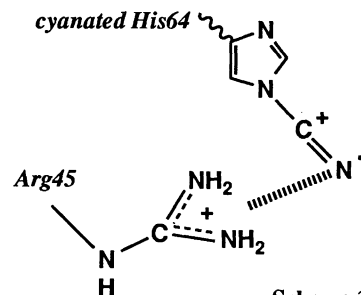
In contrast, the BrCN-modified HuMb(K45R), which was obtained by the addition of BrCN to the mutant solution, was so stable that its absorption spectrum was almost unchangeable for several ten minutes (Figure 1B). For this mutant Mb, the back-reaction of the cyanated-imidazole is extremely slow, compared with the wild-type. Further, upon addition of  $N_3^-$  to this BrCN-modified Mb, we obtained tetrazole-Mb.<sup>2</sup> All of these features for HuMb(K45R) are identical to those for SWMb. From the present study using mutant HuMb, we suggest that the electrophilic substitution reaction by BrCN (step I in Scheme 1) does occur irrespectively of the 45 residue, but the resultant cyanated form of the His64 imidazole is stabilized by Arg45 but not by Lys45. Such stabilization is closely related with the subsequent reaction of the tetrazole formation (step II in Scheme 1).

One possible explanation for the stabilization of the cyanated imidazole of His64 in HuMb(K45R) is arisen from different accessibility of the solvent water molecules to this moiety between wild-type HuMb and HuMb(K45R).<sup>4</sup> On the basis of the saturation transfer experiment, it was found that the  $H_2O$  is less accessible to His64 imidazole in HuMb(K45R) than in wild type HuMb, possibly resulting in retard of the hydrolysis of the  $>N-CN$  moiety.<sup>1</sup> Another explanation is electrostatic interaction of the guanidyl group of Arg45 with the  $-CN$  moiety. The cyanated imidazole is reasonably considered to be in a resonance structure as follows (Scheme 2);



Scheme 2.

Therefore, the interaction shown in Scheme 3 is possibly available, but is not for the  $\epsilon$ -amino group of Lys45, due to its shorter side chain. Further, it is notable that such interaction facilitates polarization of the  $-CN$  moiety, especially 2 or 3, which is convenient for the subsequent 1,3-dipole cycloaddition of  $N_3^-$  to generate the tetrazole group.



Scheme 3.

The chemical modification with BrCN and  $N_3^-$  has been strictly specified for His64 of SWMb, but has not been applicable to the distal His in other hemoproteins such as hemoglobin, in which the residue at 45 is Ser. The present study implies that our chemical technique may become feasible for the modification of the distal His of some hemoproteins in combination with recombinant DNA methods, by which we can introduce an unnatural group into proteins.

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#### References and Notes

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