Vol. 81, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1978 Pages 892-899

BROMOTRIFLUOROACETONE ALKYLATES HEMOGLOBIN AT CYSTEINE β93*

Wray H. Huestis+ and M. A. Raftery†

+Chemistry Department, Stanford University, Stanford, CA 94305

†Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125

Received February 15,1978

SUMMARY

The site of alkylation of hemoglobin by bromotrifluoroacetone has been determined by peptide mapping, using a stable radioactive label for the trifluoroacetonyl function. This study and ^{1}H and ^{19}F-nmr experiments confirm the site of modification to be the β93 cysteine found in the βT10 tryptic peptide fragment of the modified hemoglobin.

In a series of studies described previously (1, and references therein), ^{19}F-nmr** spectroscopy has been used to monitor ligand-induced conformational events in a region of the $\alpha_1\beta_2$ interface of hemoglobin. A trifluoroacetonyl (TFA) group introduced at cysteine β93 was the spectroscopic probe. Recently, Knowles (2) has questioned the selectivity of the trifluoroacetonylation reaction and suggested that the TFA label occupies several nonspecific sites in the protein. In this communication we report peptide mapping, amino acid analysis, and nmr studies which establish the site of the TFA group to be cysteine β93.

MATERIALS AND METHODS

Hb^{TFA} was prepared from hemoglobin as described (3) and labeled with ^{3}H at the alkylation site by reduction with [^{3}H]-$NaBH_4$ (New England Nuclear) (4). Globin was prepared by acid/acetone precipitation (5), taken up in water, and lyophilized. Radioactivity in the heme fraction was determined by

*The authors are indebted to Drs. Bernadine Brimhall and Richard T. Jones, University of Oregon Medical Center, for assistance in peptide mapping. This work was supported by NIH Grants HL 18660 and K04 HL 00258 (to WHH), Am 17850 (to RTJ), and GM 16424 (to MR).

**Abbreviations: nmr, nuclear magnetic resonance; Hb, hemoglobin; TFA, trifluoroacetonyl; pCMB, parachloromercuribenzoate; DTNB, 5,5'-dithiobis(2-nitrobenzoate).

0006-291X/78/0813-0892$01.00/0

liquid scintillation counting of the acetone supernatant. The whole globin was aminoethylated, digested with trypsin, and subjected to peptide mapping by column chromatography using a 10-cm column of Aminex A-5 eluted with 0.2 M pyridine-acetate buffer in a gradient from pH 3.1 to 5.0 (6,7). Fractions were collected and analyzed for radioactivity. Those fractions containing activity were rechromatographed to remove contaminants (6) and subjected to amino acid analysis. For comparison, fractions corresponding to the other cysteine-containing peptides also were rechromatographed to purity and subjected to amino acid analysis.

The trifluoroacetonyl derivative of mercaptoethanol was prepared as described (3) and the exchange rate of its α-protons was determined by ^{1}H-nmr spectroscopy using a Varian T-60 nmr spectrometer.

RESULTS AND DISCUSSION

We have shown previously that reaction of Hb with bromotrifluoroacetone blocked all cysteine sulfhydryls accessible to DTNB reaction (8). Reduction of this product (Hb^{TFA}) with [^{3}H]-$NaBH_4$ introduced a stable radioactive label which was associated irreversibly and almost quantitatively with the globin (Table 1). Separation of globin from heme, aminoethylation, and gel filtration resulted in retention of > 95% of the radioactivity in the globin. HbA reduced with [^{3}H]-$NaBH_4$ under the same conditions incorporated only about 5% as much label as Hb^{TFA}, and exhibited a similar amount of heme-labeling.

A peptide map of reduced aminoethylated Hb^{TFA} is shown in Figure 1A. The map was essentially identical to that of HbA, except that βT10 (the tryptic peptide which ordinarily contains Cys β93) was absent (6,7). A new species appeared in fractions 20-21 which contained > 90% of the radioactive label. Amino acid analyses of this peak after purification yielded a composition which compared favorably with βT10, except that the normal aminoethylcysteine was absent (Table 2). No cysteic acid or cysteine were found.

Peptides α and βT12a, which contain cysteines α104 and β112, appeared normal. The normal cleavage pattern observed for these peptides indicated that aminoethylation had been achieved, hence the cysteines could not have been oxidized or otherwise altered by the TFA modification.

These results indicate that the reaction of hemoglobin with bromotrifluoroacetone results in introduction of a borohydride-reducible moiety

TABLE 1

Specific Activity of [^{3}H]-$NaBH_4$--Reduced Hemoglobins and Fractions

Species	Activity (mCi/mmole)	
	HbA	Hb^{TFA}
Whole hemoglobin	0.32	4.44
Globin (tetramers)	0.30	4.50
Heme	0.032	0.024
Aminoethyl globin	---	4.48

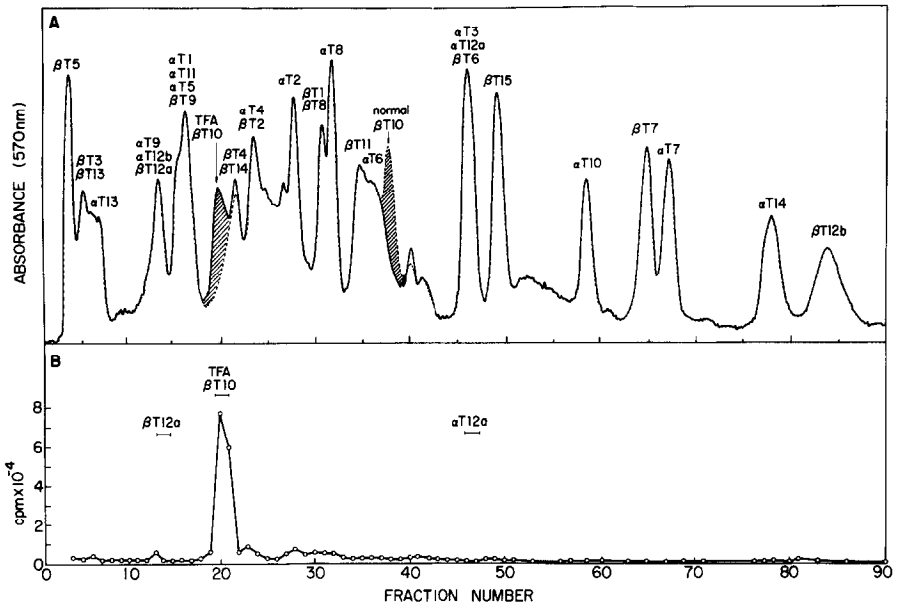


Figure 1. (A) Peptide maps of aminoethylated trypsin-digested HbA(---) and Hb^{TFA}(——). βT10 is the peptide which normally contains Cys β93. (B) ^{3}H activity in peptide fractions.

specifically at Cys β93. No evidence was found for modification or oxidation of other amino acid residues.

These findings are not in agreement with a report (2) that bromotri-

TABLE II

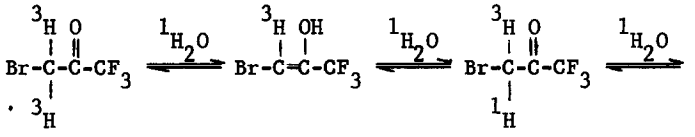
Amino Acid Compositions of Cysteine-containing Peptides from Hb^{TFA}

Amino Acid	βT10		αT12A		βT12A	
	theory	found	theory	found	theory	found
Lysine	1	1.3				
Histidine	1	1.1	1	0.9		
Aminoethyl Cysteine*	1	0	1	0.9	1	0.8
Aspartic acid	1	1.0			1	0.9
Threonine	2	1.6				
Serine	1	1.0	1	1.1		
Glutamic acid	1	0.8				
Glycine	1	0.9			1	1.5
Alanine	1	1.3				
Valine					2	2.1
Leucine	2	1.9	2	2.2	3	3.1
Phenylalanine	1	1.1				

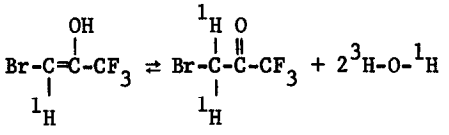
*TFA-cysteine was found to be unstable to acid hydrolysis

fluoroacetone reacts with hemoglobin in some nonspecific, noncovalent manner which results in reversible labeling of both α and β subunits and possibly of the heme. Those studies made use of bromotrifluoroacetone radioactively labeled with tritium at the α-carbon (α-^{3}H bromoTFA (I)). When Hb was allowed to react with α-^{3}H bromoTFA, normal blocking of exposed cysteines occurred but the tritium label was found randomly distributed among the globin subunits and the heme. The discrepancies between these findings and ours are best explained by a study of the exchange rates of TFA α-hydrogens under neutral aqueous conditions. The ^{1}H-nmr signal arising from bromotrifluoroacetone

(Figure 2) and from the carbonyl α-protons of S-trifluoroacetonyl mercaptoethanol (inset, Figure 2) were monitored in deuteroacetone after addition of a drop of $^{2}H_2O$. For both compounds, the magnitude of the α-proton signal diminished rapidly, with complete exchange observed in less than fifteen minutes. Under the conditions described for trifluoroacetonylation of HbA (25 minutes at 25°, pH 7.15), exchange of these protons (or of ^{3}H) by enolization should have been complete well before the protein was isolated. Thus, it is not surprising that the tritium label found associated with $\alpha\text{-}^{3}H$ Hb^{TFA} was variable (2.4 ± 0.9 moles/mole Hb tetramers) and that little label was found in globin fractions after acid/acetone precipitation (2). The label would be expected to appear at random among the H_2O-exchangeable protons of the globin and heme.

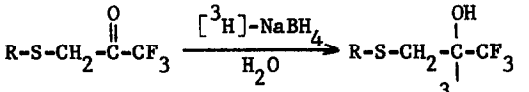


I



$$Hb\text{-}{}^{1}H + {}^{3}H\text{-}O\text{-}{}^{1}H \rightleftarrows Hb\text{-}{}^{3}H + {}^{1}H_2O$$

The borohydride reduction used in the present work produces a tritiated trifluoroisopropyl moiety (II) which is stable in aqueous solutions.



II

Another disparity between our work and that of Knowles is found in the ^{19}F-nmr properties of the two hemoglobin derivatives. We reported (3) a chemical shift for Hb^{TFA} in the range +85.0 to +85.6 ppm from $CFCl_3$

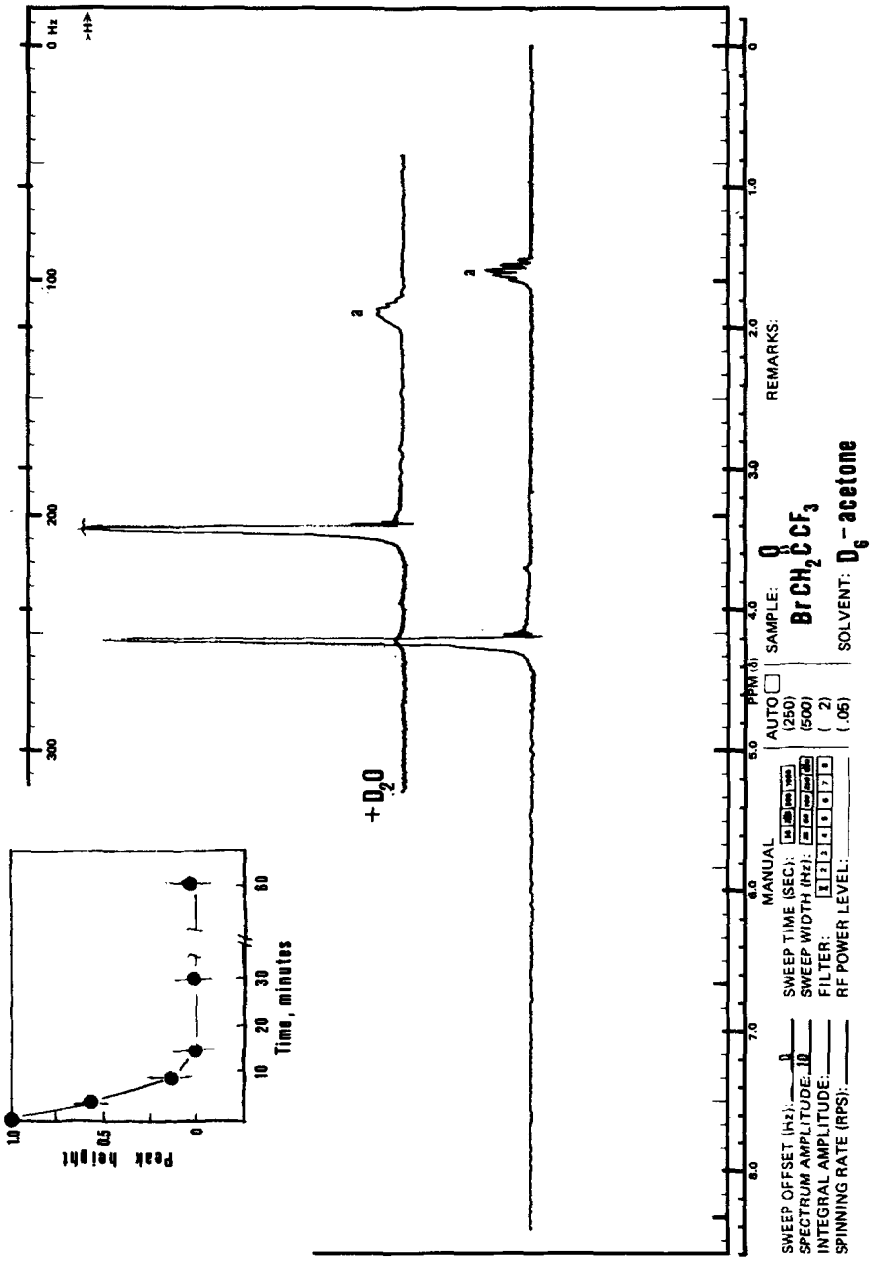


Figure 2. ^{1}H-nmr spectrum of bromotrifluoroacetone in D_6-acetone, before and 2 minutes after addition of D_2O. Signals marked "a" arise from residual ^{1}H-acetone. Inset: Time course of decrease in the proton signal (α to carbonyl) of trifluoroacetonylated mercaptoethanol, after addition of D_2O.

($\delta_{CF_3COOH} = \delta_{CFCl_3} - 79.9$ ppm). The α^3H-Hb^{TFA} used in Knowles' work exhibited ^{19}F chemical shifts in the range +112 to +113 ppm from $CFCl_3$. The literature (9) contains no precedent for trifluoroacetyl or trifluoroacetonyl groups absorbing in this chemical shift region; such chemical shifts are characteristic of aromatic fluorocarbons and other highly deshielded species (e.g. Teflon). It is clear that the nmr properties of α^3H-TFA differ radically from those of the ^{19}F probe used in our studies. Thus, its general distribution among the protein subunits is not necessarily relevant to the subunit distribution of the normal TFA label.

Several other points concerning the structure of Hb^{TFA} should be mentioned. Knowles reports observation of ^{19}F-nmr signals in α-chains separated from α^3H-Hb^{TFA} by the procedure of Geraci et al. (10). We found (Huestis and Raftery, unpublished) that a TFA group introduced by our procedure interfered with chain separation. The yields of separated chains were lower, and the α-chain fraction was seriously contaminated with hemoglobin tetramers. To prepare pure samples of Hb^{TFA} hybrids it was necessary to introduce the TFA label after removal of pCMB and recombination of the chains.

Finally, it has been suggested (2) that the masking of sulfhydryl groups observed after reaction with bromoTFA was due not to S-alkylation but to oxidation of the cysteines. A free radical mechanism was invoked in which both the heme and the sulfhydryl groups were oxidized by bromoTFA. This suggestion was supported by the observation that exposure of Hb to bromoTFA (or α^3H-bromoTFA; conditions were not stated explicitly) resulted in quantitative oxidation of the hemes after two hours. This observation is not surprising; in aqueous solution bromoTFA hydrolyses and decomposes slowly to several different fluorinated species some of which effectively denature hemoglobin. For this reason the normal alkylation procedure employs as short a reaction time as is consistent with quantitative conversion to Hb^{TFA} (20-25 min). During this time denaturing effects are minimal, and no heme oxidation can be detected (10). In addition, no cysteic acid could be detected in amino acid analyses of Hb^{TFA} prepared as described.

CONCLUSIONS

Peptide mapping of Hb^{TFA} reduced with $NaB^{3}H_{4}$ showed that the labeling site is at cysteine β93. Reports that labeling occurs on the α chains or at the heme were based on studies of a TFA analog which was labeled reversibly with tritium and which exhibited ^{19}F-nmr properties radically different from Hb^{TFA}.

REFERENCES

1. Huestis, W. H., and M. A. Raftery (1975) Biochemistry 14, 1886-1892.
2. Knowles, F. C. (1975) Biochem. Biophys. Res. Commun. 65, 1155-1162.
3. Huestis, W. H., and M. A. Raftery (1972) Biochemistry 11, 1648-1654.
4. Bode, J., M. Blumenstein, and M. A. Raftery (1975) Biochemistry 14, 1153-1160.
5. Winterhalter, K. H. and E. R. Huehns (1964) J. Biol. Chem. 239, 3699-3705.
6. Jones, R. T. (1970) Meth. Biochem. Anal. 18, 205.
7. Scheider, R. G., R. A. Hettig, M. Bilunos, and Bernadine Brimhall (1976) Hemoglobin 1, 85-96.
8. Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70-81.
9. Dungan, C. H. and J. R. vanWazer, "Compilation of Reported ^{19}F NMR Chemical Shifts" John Wiley and Sons, New York, 1970.
10. Geraci, G., L. G. Parkhurst, and Q. H. Gibson (1969) J. Biol. Chem. 244, 4664-4667.
11. Tomita, S., Y. Enoki, M. Santa, H. Loshida, and Y. Yosuminisu (1968) J. Nara Med. Assoc. 19, 1-12.