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Aspirin Acetylation of βLys-82 of Human Hemoglobin

NMR STUDY OF ACETYLATED HEMOGLOBIN TSURUMAI

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ABSTRACT. Acetylation of hemoglobin by aspirin and other compounds has been of interest for the development of agents useful for the treatment of sickle cell disease. In the present study, we have used 2D NMR methods in combination with [1-^{13}C-*acetyl*]salicylic acid to probe the acetylation sites of hemoglobin A and hemoglobin Tsurumai, a mutant human hemoglobin characterized by a βLys-82-Gln substitution. In contrast to earlier studies by Klotz and coworkers (e.g. Shamsuddin M, Mason RG, Ritchey JM, Honig GR and Klotz KM, *Proc Natl Acad Sci USA* 71: 4693–4697, 1974) in which it was concluded that βLys-144 is the principal target residue acetylated by aspirin, the present study confirms our previous but less conclusive demonstration (Xu ASL, Macdonald JM, Labotka RJ and London RE, *Biochim Biophys Acta* 1432: 333–349, 1999) that βLys-82 is the primary acetylation site of aspirin and related agents. The present studies also provide conclusive evidence that acetylation of βLys-82 produces multiple resonances, probably as a consequence of additional acetylation of other sites, particularly βLys-82′ on the second β chain. The present results also resolve the apparent discrepancy between the targets of modification by aspirin and double-headed aspirin analogs, and provide an explanation for the changes in oxygen affinity and aggregation threshold of aspirin-modified hemoglobin previously observed under *in vitro* conditions. In light of the present identification of the principal site of acetylation, the potential therapeutic benefit of aspirin in the treatment of sickle cell disease is discussed. BIOCHEM PHARMACOL **60**;7: 917–922, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. Aspirin; hemoglobin; Hb Tsurumai; sickle cell disease; NMR

Despite knowledge of the underlying genetic defect and extensive research on the pathophysiology of sickle cell disease [1], currently available treatments are of limited therapeutic value [2]. Among the various therapeutic approaches, the use of agents rationally designed to modify hemoglobin covalently or non-covalently to reduce hemoglobin polymerization remains a promising approach. One strategy in this approach is to design agents targeting residues (e.g. βVal-6, βPhe-85, and βLeu-88) located in the contact interface of the hemoglobin polymer, thereby preventing directly the formation of sickle hemoglobin fibers (e.g. Ref. 3). Binding of 2,3-DPG¶ modulates the conformational changes of hemoglobin by stabilizing the unliganded deoxy state ("T state"), thus giving rise to a decrease of oxygen affinity [4]. Thus, another attractive approach for the rational design of anti-sickling agents is to target residues in the 2,3-DPG binding cleft (the β-cleft). Such a modification prevents 2,3-DPG binding and results in a higher fraction of oxyhemoglobin, which has a reduced tendency to aggregate [1, 3]. Aspirin (acetylsalicylic acid) was one of the early agents evaluated as a covalent modifier of hemoglobin; its primary target was identified as βLys-144, located at the edge of the 2,3-DPG binding cleft, as well as other residues including βLys-59 and αLys-90, located on the surface of hemoglobin [5]. Further, HbS treated with aspirin was reported to exhibit a small increase in oxygen affinity [5, 6]. Interestingly, related "double-headed aspirin" analogs such as BDSF, which cross-link the Hb tetramer, were found to target βLys-82 rather than βLys-144 [7]. The difference in specificity between aspirin and BDSF might be explained by a more specific binding interaction of the doubly charged BDSF molecule with the 2,3-DPG binding cleft. The lack of specificity of aspirin for residues critical to sickle hemoglobin gelation has led to attempts to develop other agents that more selectively

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¶ *Abbreviations*: 2,3-DPG, 2,3-diphosphoglycerate; Hb, hemoglobin; HbS, sickle-cell Hb; MAP, methylacetyl phosphate; BDSF, bis(3,5-dibromosalicyl)fumarate; HSQC, heteronuclear-single-quantum-coherence spectroscopy; and COHbA, carbonmonoxyhemoglobin A; CNHbA, cyanomethemoglobin A; PBK, phosphate buffered KCl.

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target either the HbS–HbS interface involved in HbS polymer formation or the 2,3-DPG binding site; of these, MAP has been the most thoroughly studied ([8, 9] and references therein), and has been reported to acetylate β-globin exclusively, at three sites associated with the 2,3-DPG binding site: βVal-1, βLys-82, and βLys-144.

However, our recent NMR studies using [1′-^{13}C]aspirin and methyl-[1-^{13}C-*acetyl*]phosphate (1-[^{13}C]MAP) have indicated that the residue selectivities of these two acetylating agents toward liganded (carbonmonoxy) hemoglobin are fairly similar, with the primary acetylation site most likely being βLys-82 [10, 11]. The aspirin result is in contrast to previous studies by Klotz and co-workers [5, 6], who identified βLys-144 as the principal target for acetylation. As noted above, this conclusion would provide an attractive explanation for the observed effect of aspirin on the critical gelation threshold of hemoglobin. Aspirin is in widespread use as an anti-inflammatory agent, as an analgesic, and as a preventative agent for reducing risks for heart disease (e.g. Refs. 12 and 13) and colon cancer [14]. Hence, an unequivocal determination of the principal site(s) of aspirin acetylation of human hemoglobin is important for understanding the pharmacologic and toxicologic effects of this drug.

In our previous studies, assignment of the acetylation site(s) by NMR was achieved by analysis of the spectroscopic changes resulting from: (i) protection of hemoglobin from aspirin modification by covalent and non-covalent agents; (ii) the paramagnetic shift of the adduct resonances in cyanomethemoglobin [10]; (iii) derivatization of hemoglobin at βCys-93 using a spin label; and (iv) use of a hemoglobin triple mutant. The most unambiguous basis for assignment of the site(s) of acetylation is by comparative analysis of the adduct spectra obtained using mutant proteins [10, 15]. A hemoglobin variant with high oxygen affinity was identified recently in a 46-year-old Japanese subject [16]. Subsequent structural characterization revealed a new hemoglobin mutant, hemoglobin Tsurumai, containing a Lys → Gln mutation on the β chain at position 82 (βLys-82-Gln). The mutation at βLys-82 breaks the salt bridge between ε-NH_2 of Lys-82 and the carboxy group of 2,3-DPG, greatly weakening the binding of 2,3-DPG. The mutation also renders the residue at β-82 inert to acetylation by aspirin. To confirm unambiguously our previous assignment of βLys-82 as the primary site of aspirin acetylation, we report here our NMR analysis of aspirin-acetylated hemoglobin Tsurumai.

MATERIALS AND METHODS

Blood samples were collected from the donor, and the erythrocytes were washed in 5 vol. of physiological saline prior to being frozen at −80°. Since the mutant hemoglobin comprises only 49.4% of the total hemoglobin [16], the mutant hemoglobin was purified by DEAE–cellulose anion exchange chromatography using a procedure reported previously [5]. Following the protocols used in our earlier studies, the purified hemoglobin then was reacted with a 10-fold molar excess of [1′-^{13}C]aspirin at 37° for a 12-hr period. The acetylation reaction was terminated, and the sample was dialyzed immediately against cold 20 mM potassium phosphate-buffered saline to remove the remaining [1′-^{13}C]aspirin. The dialyzed adduct hemoglobin was concentrated by membrane filtration prior to the NMR study. The acetylation of cyanomet hemoglobin (CNHbA) was carried out following a protocol similar to that reported previously [10], except that the reaction was allowed to continue for 12 hr at 37°. The acquisition and processing of the 2D ^{1}H-^{13}C HSQC spectra of hemoglobin adducts were performed following protocols reported earlier [10].

RESULTS AND DISCUSSION

Aspirin is known to acetylate hemoglobin, targeting primary amino groups in the 2,3-DPG binding cleft and several hemoglobin surface residues [5, 10]. Two-dimensional ^{1}H-^{13}C HSQC/HMQC NMR analysis allows the identification of multiple adduct resonances simultaneously without the need for isolation and enzyme digestion of the adducts, thus minimizing potential errors in analysis of minor protein adducts. However, assignment of the adduct resonances of large proteins often poses a difficult challenge, which may require a variety of approaches, as demonstrated previously [10, 15]. The observed spectra correspond to a mixture of singly and multiply acetylated species, and a stoichiometric analysis of the mixture is not performed readily. Figure 1 shows the 2D ^{1}H-^{13}C HSQC spectra of [1′-^{13}C]aspirin-acetylated COHbA and carbonmonoxyhemoglobin Tsurumai. As is apparent from this figure, the mutation of βLys-82 to glutamine in hemoglobin Tsurumai results in the loss of a set of intense resonances including peaks labeled 5, 6, and 9 in the acetylated COHbA. The absence of resonances 5, 6, and 9 in the adduct of hemoglobin Tsurumai allows their unambiguous assignment to acetyl-βLys-82, thus confirming our previous assignment based on kinetic data, site protection, and analysis of the paramagnetic shift of adduct resonances [10]. The remaining adduct resonances appear to be similar between the acetylated COHbA and COHb-Tsurumai, indicating that the single mutation does not introduce significant perturbation of the structure near these acetylated residues.

Our recent NMR studies of protein adducts have indicated that at intermediate levels of adduct formation, a single acetylated residue can give rise to multiple resonances [10, 15]. This arises if the chemical shift of a particular adduct peak is perturbed by formation of additional adducts nearby. The position of βLys-82 in the hemoglobin A β-cleft suggests that this residue will be particularly susceptible to such effects due to its proximity to other modification sites—particularly the neighboring βLys-82′ on the second β chain (Fig. 2). As discussed previously, dependence of the resonance intensities on the duration of incubation with [1′-^{13}C]aspirin suggested that

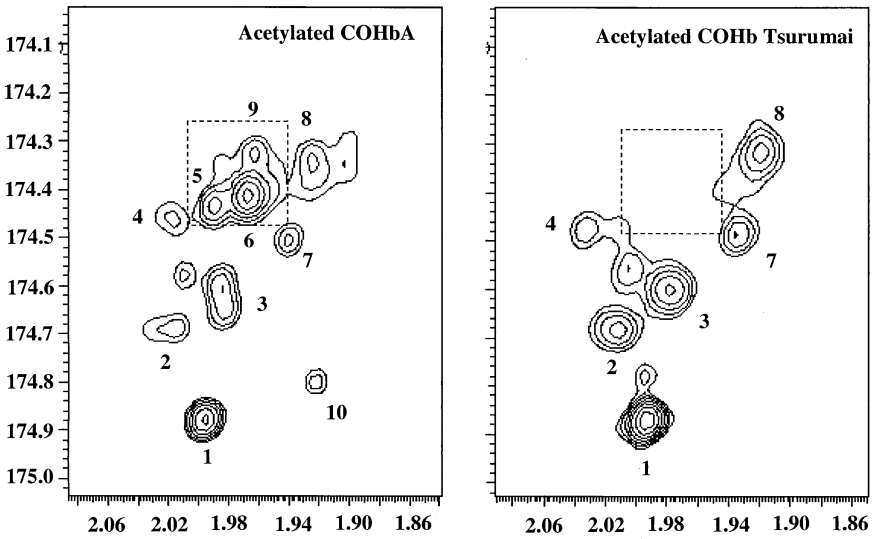

FIG. 1. ^{1}H-^{13}C HSQC NMR spectra of COHbA and Hb Tsurumai exposed to [1′-^{13}C]aspirin for a period of 12 hr at 37°. Samples contained 1.29 and 2.58 mM acetylated carbonmonoxy hemoglobin A and carbonmonoxy hemoglobin Tsurumai in 20 mM phosphate-buffered KCl (140 mM, pH 7.1). The spectra were acquired and processed using the protocol described previously [10, 11].

peak 6, formed initially, corresponded to an acetyl-βLys-82 adduct, whereas peak 9, which formed more slowly, was proposed to correspond to the double adduct: acetyl-βLys-82 and acetyl-βLys-82′ hemoglobin A. The shift from the singly modified acetyl-βLys-82 resonance might be due to the elimination of the nearby positive charge. Resonance 5 was also proposed to correspond to βLys-82 arising from additional (unknown) adduct formation, e.g. at βVal-1 or βLys-144.

Further support for this interpretation was derived from studies of cyanomethemoglobin adducts [10]. Formation of the paramagnetic cyanomet derivative results in shifts of the ^{1}H and ^{13}C resonances, which depend on the orientation of the acetyl adduct relative to the g-tensor of the heme iron. Correlations of the principal resonances of the carboxyhemoglobin and cyanomethemoglobin adducts are based on the theoretical form of the expression for dipolar shifts and the use of the approximation that for adducts sufficiently far from the paramagnetic center, the orientations of the ^{13}C and ^{1}H nuclei relative to the heme are approximately constant for each acetyl group. Thus, the resulting dipolar shifts will obey the relation $\Delta^{13}C \sim \Delta^{1}H$ if the shifts are expressed in ppm. The validity of this assumption is demonstrated more readily for less extensively acetylated hemoglobin, in which only a few resonances need to be correlated [10]. In this case, the group of

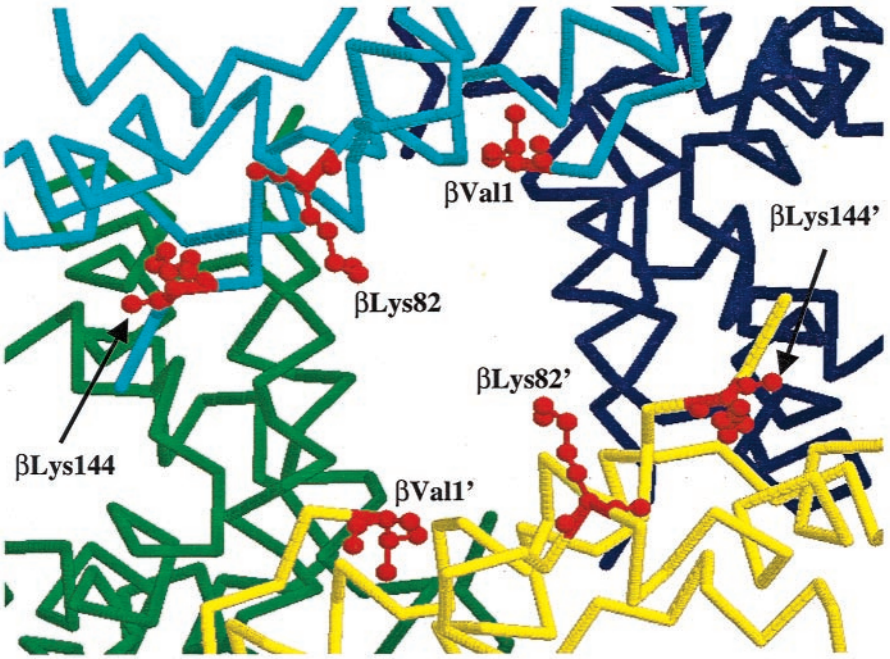

FIG. 2. The β-cleft region of oxyhemoglobin A showing the βVal-1, βLys-82, and βLys-144 residues, which have been reported to be modified by acetylating agents. The structure is based on the x-ray crystallographic structure of 1HGC [17] and is used for the purpose of illustrating the relative positions of βLys-82 and βLys-82′.

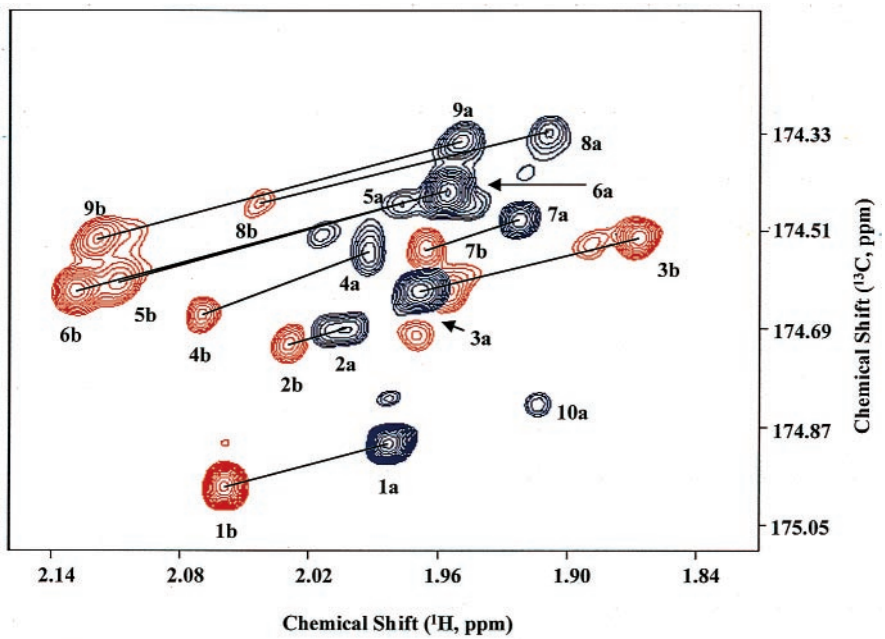

FIG. 3. Overlaid ^{1}H- ^{13}C HMQC spectra of 1.2 mM acetylated carbonmonoxy hemoglobin A and cyanomet hemoglobin A in 20 mM PBK (pH 7). The hemoglobin was acetylated by reaction with a 10-fold excess of [1′-^{13}C] aspirin at 37° for 12 hr. The spectra were acquired and processed similarly to those of Fig. 1. The two spectra are overlaid to highlight the changes of chemical shifts of the adducts resulting from the paramagnetic heme. The resonances are labeled following the same convention used in Fig. 1, with suffixes 'a' or 'b' to identify peaks in carbonmonoxy hemoglobin and cyanomet hemoglobin adducts, respectively.

resonances corresponding to peaks 5, 6, and 9 was observed to shift together (Fig. 3), again suggesting that they might arise from modification of a single residue or set of equivalent residues. Hence, the present data obtained for aspirin-modified hemoglobin Tsurumai are consistent not only with the assignment of βLys-82 as the primary target of aspirin acetylation, but also with the conclusion that this acetylated residue corresponds to multiple peaks.

The present studies show conclusively that aspirin can target the βLys-82 residue of hemoglobin effectively as the primary site of modification. Since this residue is involved in 2,3-DPG binding [4, 18], acetylation of βLys-82 will strongly reduce the affinity of 2,3-DPG for hemoglobin. Thus, the results of this study provide an explanation for the ability of aspirin to increase the oxygen affinity of deoxyHbS [6], and resolve the apparent inconsistency between the target residues of mono- and bi-functional aspirin derivatives reported in early studies [5, 7, 19]. The assignment is consistent with our recent NMR analyses [10], which, however, were less conclusive in the absence of a comparison with hemoglobin mutated at position 82. This gap is now filled by the present study using hemoglobin Tsurumai. Although other hemoglobin residues are also modified by aspirin, the degrees of such modification may be relatively modest despite the high concentration of aspirin and the extended reaction time used in the present study.

As proposed originally by Walder and co-workers [20], the primary criteria for rational design of anti-sickling chemotherapeutic agents are the following: (i) the agents should reduce significantly the polymerization of hemoglobin; (ii) the agents should be specific for hemoglobin, with minimal effect on other proteins to minimize potential cytotoxic effects; (iii) the oxygen binding affinity of modified hemoglobin should not differ drastically upon chemical modification; (iv) the agents should be membrane-permeable to allow uptake into erythrocytes. To what extent does aspirin satisfy Walder's criteria? Aspirin and several of its metabolites have been shown to penetrate the erythrocyte membrane rapidly, reaching maximum concentration within 1 min [21]. *In vitro* studies of hemolysates from aspirin-treated normal erythrocytes showed normal oxygen affinity, whereas the purified hemoglobin A from those erythrocytes had a slightly increased affinity. Klotz and Tam [6], in an *in vitro* study of aspirin acetylation of hemoglobin S and sickle cells, have also found acetylation to increase the oxygen affinity slightly, which has been proposed to result from blockage of 2,3-DPG binding.

However, the action of aspirin is not specific for hemoglobin. *In vivo*, aspirin primarily targets cyclooxygenase as a nonsteroidal anti-inflammatory drug [22] in addition to its acetylation of other proteins such as hemoglobin and serum albumin [5, 23]. There is also evidence for modification of erythrocyte membrane proteins by aspirin [24]. It is evident, under *in vitro* conditions, that significant acetylation of hemoglobin requires prolonged reaction with a high dose of aspirin [10]. The level of aspirin normally achieved *in vivo* is about 0.2 mM [25]. Furthermore, the half-life of aspirin in the bloodstream is estimated to be ~22 min [26]. Interestingly, this is due largely to the erythrocytes themselves,

which have been shown to contain aspirin esterase [25]. Similar studies of "double-headed" aspirin and analogues showed that competing hydrolysis of the reagents by enzymes on the erythrocyte outer membrane may limit their bioavailability for reaction with erythrocytes [27]. Therefore, a significant degree of hemoglobin acetylation may be difficult to achieve *in vivo*, and likely would require frequent and prolonged exposure to the drug, with its attendant toxic effects. In one study of healthy volunteers on long-term, high-dose aspirin therapy, the level of modified hemoglobin was found to be about 4–5% of the total hemoglobin [28].

Aspirin is among the most extensively used drugs; its efficacy and toxicity have been evaluated over the years. It has been prescribed for long-term use in the treatment of arthritis [29] and the prevention of heart diseases (e.g. Refs. 12 and 13) and more recently colon cancer [14]. However, early clinical studies on the therapeutic effect of aspirin in sickle cell disease have been controversial as a result of the large variation of the experimental conditions under which those studies were performed. In an uncontrolled clinical observation, Osamo *et al*. [30] reported significant therapeutic benefit, ascribed to the increase of oxygen affinity and life span of the sickle cells, suggesting possible use in treatment of sickle cell disease. However, other investigations did not support this conclusion [31–33]. These studies were motivated by the use of aspirin as a platelet inhibitor and analgesic agent for management of sickle pain crisis rather than for hemoglobin modification, and in no case was the degree of hemoglobin modification evaluated to correlate with overall oxygen affinity and other clinical observations. It is likely that the discrepancy between *in vitro* studies and clinical observations might be attributed to the insufficient acetylation of hemoglobin *in vivo* because of the low bioavailability of the drug. Although long-term use of aspirin is likely to be of limited benefit in sickle cell disease, knowledge of the sites of action responsible for the anti-sickling effects of covalent modifiers should lead to the development of agents that are more specific and have higher bioavailability.

In conclusion, the present study shows that aspirin acetylates hemoglobin primarily at the residues in the 2,3-DPG binding cleft with βLys-82 as the principal target. This further supports our recent assignment using a range of other assignment strategies [10], resolves the apparent discrepancy between the targets of modification by aspirin and double-headed aspirin analogs, and provides an explanation for the changes in oxygen affinity and aggregation threshold of aspirin-modified hemoglobin observed under *in vitro* conditions.

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