Acetylation of Human Hemoglobin by Methyl Acetylphosphate

EVIDENCE OF BROAD REGIO-SELECTIVITY REVEALED BY NMR STUDIES*

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The development of chemical modification agents that reduce the tendency of sickle hemoglobin (HbS) to aggregate represents an important chemotherapeutic goal. Methyl acetylphosphate (MAP) has been reported to bind to the 2,3-diphosphoglycerate (2,3-DPG) binding site of hemoglobin, where it selectively acetylates residues, resulting in increased solubility of HbS. We have prepared [1-13C]MAP and evaluated the adduct formation with hemoglobin using 1H-13C HMQC and HSQC NMR studies. These spectra of the acetylated hemoglobin adducts showed 10–11 well resolved adduct peaks, indicating that the acetylation was not highly residue specific. The chemical shift pattern observed is in general similar to that obtained recently using [1-13C]aspirin as the acetylation agent (Xu, A. S. L., Macdonald, J. M., Labotka, R. J., and London, R. E. (1999) Biochim. Biophys. Acta 1432, 333–349). Blocking the 2,3-DPG binding site with inositol hexaphosphate (IHP) resulted in a selective reduction in intensity of adduct resonances, presumably corresponding to residues located in the 2,3-DPG binding cleft. The pattern of residue protection appeared to be identical to that observed in our previous study using IHP and labeled aspirin. Pre-acetylation of hemoglobin using unlabeled MAP, followed by acetylation with [1-13C]aspirin indicated a general protective effect, with the greatest reduction of intensity for resonances corresponding to acetylated residues in the 2,3-DPG binding site. These studies indicated that both MAP and aspirin exhibit similar, although not identical, acetylation profiles and target primarily the βLys-82 residue in the 2,3-DPG binding site, as well as sites such as βLys-59 and αLys-90, which are not located in the β-cleft of hemoglobin.

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Therapeutic approaches for the treatment of sickle cell disease include induction of fetal hemoglobin expression, bone marrow transplantation, and the use of pharmacological agents that interact either non-covalently or covalently with HbS¹ to inhibit aggregation (1). The latter approach remains a potentially important therapeutic strategy, which has been under exploration by a number of groups (2–6), although to date no agents of this class have been successfully developed for clinical use. Since the first report of hemoglobin carboxylation by cyanate as a potentially effective anti-sickling agent (7), a number of other agents have been designed, and their suitability as anti-sickling drugs has been investigated (e.g. Refs. 5 and 8). Among these reagents is methyl acetylphosphate (MAP), an acetylating agent that was first designed as an inhibitor of n-3-hydroxybutyrate dehydrogenase (9). Based on the investigation of the modification reaction using conventional protein chemistry techniques, MAP was reported to target residues in the 2,3-DPG binding site: βLys-144, βLys-82, and βVal-1 (Refs. 10 and 11 and references therein). No residues of the α-chain were reported modified in the same study (10). The modification of HbS by MAP was reported to yield adduct hemoglobin with oxygen affinity similar to that of hemoglobin A but with a reduced gelation tendency. The decrease in the affinity of 2,3-DPG and in gelation tendency is thought to result from the MAP selective acetylation of amino groups located in the β-cleft to which 2,3-DPG binds. Among the set of criteria first proposed by Walder et al. (3) for a suitable anti-sickling agent, specificity for selected target residues of hemoglobin is of critical importance in order to limit the introduction of undesired structural and functional changes of the hemoglobin as well as the toxicity of the agent resulting from modifications of other proteins. Multidimensional NMR in conjunction with isotopically labeled agents has been shown to be an effective approach for studying chemical modification of hemoglobin (12) and ubiquitin (13). We report here our NMR study of the sites/residues of human hemoglobin acetylated by MAP. Results from this study are important for evaluating the effects of MAP acetylation of hemoglobin and hence are useful for the evaluation of this compound as a possible anti-sickling agent.

EXPERIMENTAL PROCEDURES

Inositol hexaphosphate sodium salt (IHP) was from Calbiochem-Novabiochem; salicylic acid was from Fluka (Ronkonkoma, NY); and Slide-A-Lyzer dialysis membrane cassettes (molecular mass cut-off, 10 kDa) were obtained from Pierce. Centriprep and Centricon membrane concentrators were from Amicon (Beverly, MA). All other reagents were of analytical grade. Human hemoglobin A was prepared using the protocol reported in our previous study (12). [1-13C]Aspirin was synthesized using the procedures reported elsewhere (12, 13). [1-13C]Methyl acetylphosphate was prepared by a reported synthesis procedure (14) using [1-13C]acetyl bromide as the precursor for isotopic labeling.

The acetylation of the e-NH₂ of lysine and α-NH₂ of N-terminal residues of hemoglobin by [1-13C]MAP was carried out under the following conditions: hemoglobin (typically 2 mM) in either carbonmonoxygen

¹ The abbreviations used are: HbS, sickle hemoglobin; MAP, methyl acetylphosphate; IHP, inositol hexaphosphate; 2,3-DPG, 2,3-diphosphoglycerate; PBK, –35 mm phosphate and 140 mm KCl, pH 7; HMQc, heteronuclear multi-quantum coherence spectroscopy; HSQC, heteronuclear single-quantum coherence spectroscopy; 2,3-DPG, 2,3-diphosphoglycerate; COHbA, carbonmonoxyhemoglobin A; OxyHbA, oxyhemoglobin A; CNHbA, cyanomethemoglobin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
or oxy form (OxyHbA) where indicated was allowed to react with a 10-fold molar excess of MAP in phosphate-buffered saline (PBK; 35 mM phosphate, 140 mM KCl, pH 7) at 37 °C for 1 h. At the completion of the incubation, the hemoglobin sample was dialyzed immediately against a 1000-fold excess volume of cold PBK (20 mM phosphate) at 4 °C overnight, with two to three changes of buffer to quench the reaction and to remove the unreacted MAP and acetate. The dialyzed hemoglobin adduct solutions were then reconcentrated for the NMR experiments using a Slide-A-Lyzer membrane microconcentrator. Total hemoglobin concentration was determined by measuring the concentration of cyanomethemoglobin (CNHbA) using the Total Hemoglobin kit from Sigma. Acetylation of hemoglobin by aspirin was achieved under similar conditions except that the reaction was allowed to proceed for 12 h prior to dialysis. The HMQC (15) and HSQC (16) experiments were adapted to acquire the spectra of the chemically modified hemoglobin adducts. Details of spectral acquisition, processing, and analysis are reported elsewhere (12).

RESULTS AND DISCUSSION

In this study, 1-13C-labeled methyl acetylphosphate was synthesized and used to acetylate human hemoglobin. The presence of the isotopic label allowed detection of the adduct resonances of large proteins such as hemoglobin using 1H-13C HMQC or HSQC spectroscopy. Detection utilizing the relatively weak polarization transfer between the carbonyl 13C and methyl protons of the acetyl group was facilitated by the high mobility of the acetyllysine side chains, and the carbonyl resonances were found to yield greater shift dispersion than the methyl resonances observed using 2-13C]acetyl-labeled precursors. The 1H-13C HMQC spectrum of COHbA acetylated using a 10-fold molar excess of [1-13C]MAP:COHbA, t1 data points and 128 complex t2 data points, with 16 scans per t1 increment. A 90° shifted nine-squared function was applied in both dimensions, and the data were zero-filled to 4096 × 2048 points prior to Fourier transformation. The 1H and 13C chemical shifts were set to 0.0 and −2.0 ppm for 3-(trimethylsilyl)-1-propanesulfonic acid. Use of a small spectral window to improve the 13C spectral resolution resulted in folding of the [1-13C]acetate resonance at −182 ppm into the region of interest. The peaks are labeled from 1 to 11 arbitrarily for identification purposes. Ace, denotes the resonances of [1-13C]acetate.

FIG. 1. A, 1H-13C HMQC spectra of 2 mM COHbA acetylated using a 10-fold excess of [1-13C]MAP in 35 mM potassium phosphate-buffered KCl (140 mM, pH 7), PBK, at 37 °C for 1 h. The spectra were acquired at 37 °C with 2048 t1 data points and 128 complex t2 data points, with 16 scans per t1 increment. A 90° shifted nine-squared function was applied in both dimensions, and the data were zero-filled to 4096 × 2048 points prior to Fourier transformation. The 1H and 13C chemical shifts were set to 0.0 and −2.0 ppm for 3-(trimethylsilyl)-1-propanesulfonic acid. Use of a small spectral window to improve the 13C spectral resolution resulted in folding of the [1-13C]acetate resonance at −182 ppm into the region of interest. The peaks are labeled from 1 to 11 arbitrarily for identification purposes. Ace, denotes the resonances of [1-13C]acetate.

FIG. 2. Spectral intensities of [1-13C]MAP acetylation adducts of OxyHbA as a function of the [1-13C]MAP:HbA concentration ratio. OxyHbA in phosphate-buffered KCl (140 mM), pH 7, was reacted with a 10-fold molar excess of [1-13C]MAP at 37 °C for 1 h. The reacted OxyHbA was converted to COHbA prior to acquisition of the 1H-13C HSQC spectra. The two-dimensional data were processed similarly to those described for Fig. 1A.
acetylated before significant modification of less reactive sites. Hence, these data also indicate that although there is significant variation in the rates of acetylation of the various hemoglobin residues, these differences are not sufficient to achieve a high degree of residue selectivity.

Assignment of the adduct resonances in acetylated hemoglobin represents a difficult problem. We have recently performed extensive studies on hemoglobin acetylated with \([1^{13}C]\)aspirin (12) and assigned the resonances using a broad range of approaches. These included protection of the 2,3-DPG binding site with both covalent and non-covalent agents, modification of \(\beta\text{Cys-93}\) with a spin label, the use of site-directed hemoglobin mutants, and prediction of dipolar shifts in paramagnetic \(\text{CNHbA}\). These studies have allowed assignments of a number of the adduct resonances and also demonstrate that the most highly modified residue is \(\beta\text{Lys-82}\) rather than \(\beta\text{Lys-144}\) as previously reported (17). The acetyl \(\beta\text{Lys-82}\) adduct has been shown to give rise to several resonances (12). The latter phenomenon occurs in more highly modified hemoglobin, apparently as a result of the additional modification of nearby residues leading to a chemical shift inequivalence (12). In general, the HMQC spectra of hemoglobin acetylated using \([1^{13}C]\)aspirin or \([1-^{13}C]\)MAP were fairly similar, indicating that \([1^{13}C]\)aspirin and \([1-^{13}C]\)MAP share major adduct sites. Indeed, our recent studies (12) and investigations by Manning and co-workers (Ref. 10 and reference therein) show that both agents acetylate \(\beta\text{Lys-82}\) in the 2,3-DPG binding pocket (10, 12). Based on this spectral comparison and in light of our recent study of aspirin acetylation (12), we assign resonances 1, 3, and 6 to acetyl \(\beta\text{Lys-59}\), acetyl \(\alpha\text{Lys-90}\), and acetyl \(\beta\text{Lys-82}\), respectively. Resonance 8 is tentatively assigned to acetyl \(\beta\text{Lys-144}\). Further, as in the aspirin study, it is likely that resonance 9 arises from hemoglobin molecules acetylated at both the \(\beta\text{Lys-82}\) and \(\beta\text{Lys-82}\) on the second \(\beta\)-chain, because the latter represents the potential acetylation site nearest to \(\beta\text{Lys-82}\). Resonance 5 may also arise from acetyl \(\beta\text{Lys-82}\) in hemoglobin, which is additionally acetylated at other nearby or interacting sites. This conclusion is supported by several lines of evidence, particularly the identical shifts observed for resonances 6 and 9 in acetylated paramagnetic cyanomethemoglobin (12).

To confirm the assignment of acetyl \(\beta\text{Lys-82}\) and other adduct residues in the 2,3-DPG pocket, acetylation of hemoglobin with \([1-^{13}C]\)MAP was carried out in the presence of a 1–4-fold molar excess of IHP. IHP was utilized to block access to the 2,3-DPG binding site of COHbA because of its greater affinity for this site (\(K_D = 10^{-4}\) M) (18), compared with 2,3-DPG (\(K_D = 2.4 \times 10^{-2}\) M) (19). The binding of IHP resulted in a substantial decrease in intensity for peaks 6, 8, and 9 (Fig. 3), consistent with the assignment of these resonances to acetyl \(\beta\text{Lys-82}\) and acetyl \(\beta\text{Lys-144}\) in the 2,3-DPG binding pocket. However, no significant decreases of the intensities of peaks 1, 2, 3, 4, 7, 10,
and 11 were observed, indicating the high selectivity of the protective effect of IHP against subsequent acetylation by [1-13C] MAP. Thus, the lack of a significant reduction in the intensities of peaks 1, 2, 3, 4, 7, 10, and 11 (Fig. 3) indicates that the corresponding residues are located outside of the 2,3-DPG binding pocket. This result appears to be in direct contrast to the early report by Manning and co-workers (Ref. 10 and references therein), according to which no acetylation of residues outside the 2,3-DPG binding site was noted.

To further compare the specificities of aspirin and MAP as acetylating agents, COHbA was exposed initially to a 10-fold molar excess of unlabeled MAP, which was followed by exposure to [1-13C]aspirin. The HMQC spectra of COHbA acetylated with and without prior exposure to unlabeled MAP are shown in Fig. 4. The adduct spectrum corresponding to [1-13C]aspirin-acetylated COHbA (Fig. 4A) has been labeled with primes to distinguish the resonances from the adduct spectra derived from [1-13C] MAP treatment of the hemoglobin. A comparison of Figs. 1A and 4A indicates that very similar adduct species were generated, as discussed above. A comparison of Fig. 4, panels A and B, reveals a substantial decrease in intensity of the adduct resonances 5, 6, 9, and 8 shown in Fig. 2. These results indicate that both acetylating agents appear to be capable of targeting a similar residue located outside of the 2,3-DPG binding pocket. This result appears to be in direct contrast to the early report by Manning and co-workers (Ref. 10 and references therein), according to which no acetylation of residues outside the 2,3-DPG binding site was noted.

In summary, acetylation of oxyHbA or COHbA with methyl acetylphosphate under the conditions used in previously reported studies (4–6, 10, 20, 21), as well as in a phosphate buffer, yields HMQC spectra with 10–11 adduct resonances. In general, the pattern of acetylation appears to be similar to that obtained using [1-13C] aspirin (12). Observation of the resonance intensities at various MAP:COHbA ratios indicates a preference for acetylation of βLys-82, located in the β-cleft involved in the binding of 2,3-DPG.

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