Establishment of the enzymatic protein acetylation independent of acetyl CoA: recombinant glutathione *S*-transferase 3-3 is acetylated by a novel membrane-bound transacetylase using 7,8-diacetoxy-4-methyl coumarin as the acetyl donor

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Abstract The current knowledge on biological protein acetylation is confined to acetyl CoA-dependent acetylation of protein catalyzed by specific acetyl transferases and the non-enzymatic acetylation of protein by acetylated xenobiotics such as aspirin. We have discovered a membrane-bound enzyme catalyzing the transfer of acetyl groups from the acetyl donor 7,8-diacetoxy-4-methyl coumarin (DAMC) to glutathione *S*-transferase 3-3 (GST3-3), termed DAMC:protein transacetylase (TAase). The purified enzyme was incubated with recombinant GST3-3 subunit and DAMC, the modified protein was isolated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in gel digested with trypsin and the tryptic digest was analyzed by mass spectrometry. The N-terminus and six lysines, Lys-51, -82, -124, -181, -191 and -210, were found to be acetylated. The acetylation of GST3-3 described above was not observed in the absence of either DAMC or TAase. These results clearly establish the phenomenon of protein acetylation independent of acetyl CoA catalyzed by a hitherto unknown enzyme (TAase) utilizing a certain xenobiotic acetate (DAMC) as the active acetyl donor.

Key words: Acetyl donor; Protein acetylation; Matrix-assisted laser desorption/ionization-time of flight; Transacetylase

1. Introduction

Transacetylase is the enzyme that transfers acetyl groups from an acetylated compound, either a physiological metabolite or a xenobiotic, to a receptor molecule. The familiar examples of such transacetylases are: (a) the enzyme catalyzing the transfer of acetyl groups from platelet activating factor (1-alkyl-2-acetyl phosphatidyl choline) to sphingosine [1,2]; (b) the enzymatic transfer of acetyl groups from *N*-acetyl-arylamine to an amide [3]. Proteins are also the targets for enzymatic acetylation. The acetyl CoA-dependent enzymatic acetylation of histones catalyzed by histone acetyl transferases (HAT) stands prominent [4], but the knowledge on protein acetylation independent of acetyl CoA is grossly confined to be non-enzymatic in nature. The most familiar example is the observation of Sir John Vane that aspirin (acetyl salicylate) acetylates cyclooxygenase resulting in inhibition of prostaglandin synthesis [5]. The enzymatic protein acetylation, independent of acetyl CoA, has not been reported up to date. The existence of a unique enzyme in rat liver microsomes that catalyzes the transfer of acetyl groups from acetoxy xenobiotics to the specific proteins was demonstrated for the first time in our previous communications [6–8]. Acetoxy-4-methyl coumarins were found to be versatile substrates for the enzyme termed DAMC:protein transacetylase (TAase) catalyzing the possible acetylation of some specific enzyme proteins viz. cytochrome *P*-450, NADPH cytochrome *c* reductase and glutathione *S*-transferase (GST) leading to modulation of their catalytic activities. DAMC served as a model acetyl group donor and GST as acetyl group receptor protein. A convenient assay procedure was developed for TAase [8] based on the irreversible inhibition of GST, the extent of the inhibition of GST was considered as a measure of DAMC:GST TAase activity. The TAase-mediated acetylation of GST3-3 (isoform of GST) reported here unravels the hitherto unknown phenomenon of protein acetylation independent of acetyl CoA. In addition, TAase-mediated acetylation would amount to protein modification and hence the selective specificity of acetylated drug may be utilized to target-desired protein resulting in the expression of desired pharmacological activity.

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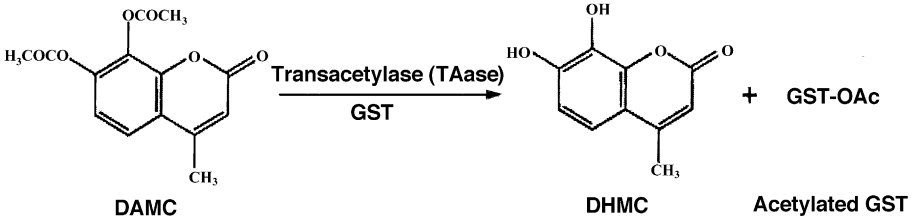


Fig. 1. TAase-catalyzed reaction.

2. Materials and methods

2.1. Demonstration of acetylation of GST by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS)

The reaction mixture containing TAase (50 μg), recombinant GST3-3 isoform (50 μg) and DAMC (200 μM) in 0.25 M potassium phosphate buffer (pH 6.5) was incubated for 30 min at 37°C and the reaction was subjected to SDS–PAGE in order to separate modified GST3-3 from other contaminating proteins. The reaction mixture with DMSO served as the control. The gel plugs (modified and unmodified GST3-3) were separately washed and treated with dithiothreitol (DTT) and iodoacetamide to alkylate the cysteine residues present in the proteins. After reduction and alkylation, the gel plugs were incubated with sequence grade trypsin overnight [9]. The peptide mixture obtained after trypsin digestion was diluted four times with matrix 2,5-dihydroxy benzoic acid (20 mg/ml) in 1:1 acetonitrile/0.1% TFA v/v 1 μl of the mix, and deposited on a MALDI target and allowed to dry in order to perform MALDI-TOF MS analysis using Bruker Biflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser emitting pulsed ultraviolet light at 337 nm and operated in a reflectrone mode. External linear calibration was achieved using a mixture of angiotensin II, substance P, bombesin adrenocorticotropic hormone (fragment 18–39) and somatostatin (all from Sigma Chemical Company). Spectra were acquired in the positive ion mode and the signal was accumulated and averaged over 100–200 shots.

2.2. Liquid chromatography (LC)/MS/MS

LC/MS/MS measurements were conducted on an LCQ DECA (Thermoquest, San Jose, CA, USA) using a conventional ESI source in positive ion mode detection. The spray voltage was set at 3.8 kV and the heated capillary temperature at 300°C. The eluent flow of 25 μl/min was provided by an Eldex micro LC (separations). Analyses were conducted on a 15 cm × 800 μm i.d. column packed with 5 μm spherisorb C_{18} reverse phase material (LC Packings, Amsterdam, The Netherlands). The injection volume was 10 μl. Gradient elution was performed by using the following mobile phases: A: 10 mM ammonium acetate in 0.1% HCOOH (v/v); B: 10 mM ammonium acetate in 0.15% HCOOH (v/v) and 80% acetonitrile (v/v). Gradient: from 5 to 30% B in 25 min, from 30 to 60% B in 10 min, from 60 to 100% B in 5 min, down to 5% B again in 2 min. Two full-scan (ranged *m/z* 200–2000) LC-MS runs were initially acquired for modified and non-modified GST. Potentially acetylated peptides previously detected by MALDI-TOF experiments were found back in LC-MS total ion current (TIC) of the modified GST as singly or doubly protonated molecules, though their signal intensity in full-scan MS was rather weak compared to other peptides present in the digest mixture. A second LC-MS experiment with acquisition windows was designed in order to perform MS/MS on these seven potentially acetylated peptides. In this second LC-MS experiment, the mass spectrometer program was switched to MS/MS mode in correspondence to the retention time of the above-mentioned peaks. Precursor ions were selected with window of 2.5 *m/z*. The facilities for MALDI-TOF and LC/MS established at TNO Voeding, Zeist (The Netherlands) were utilized.

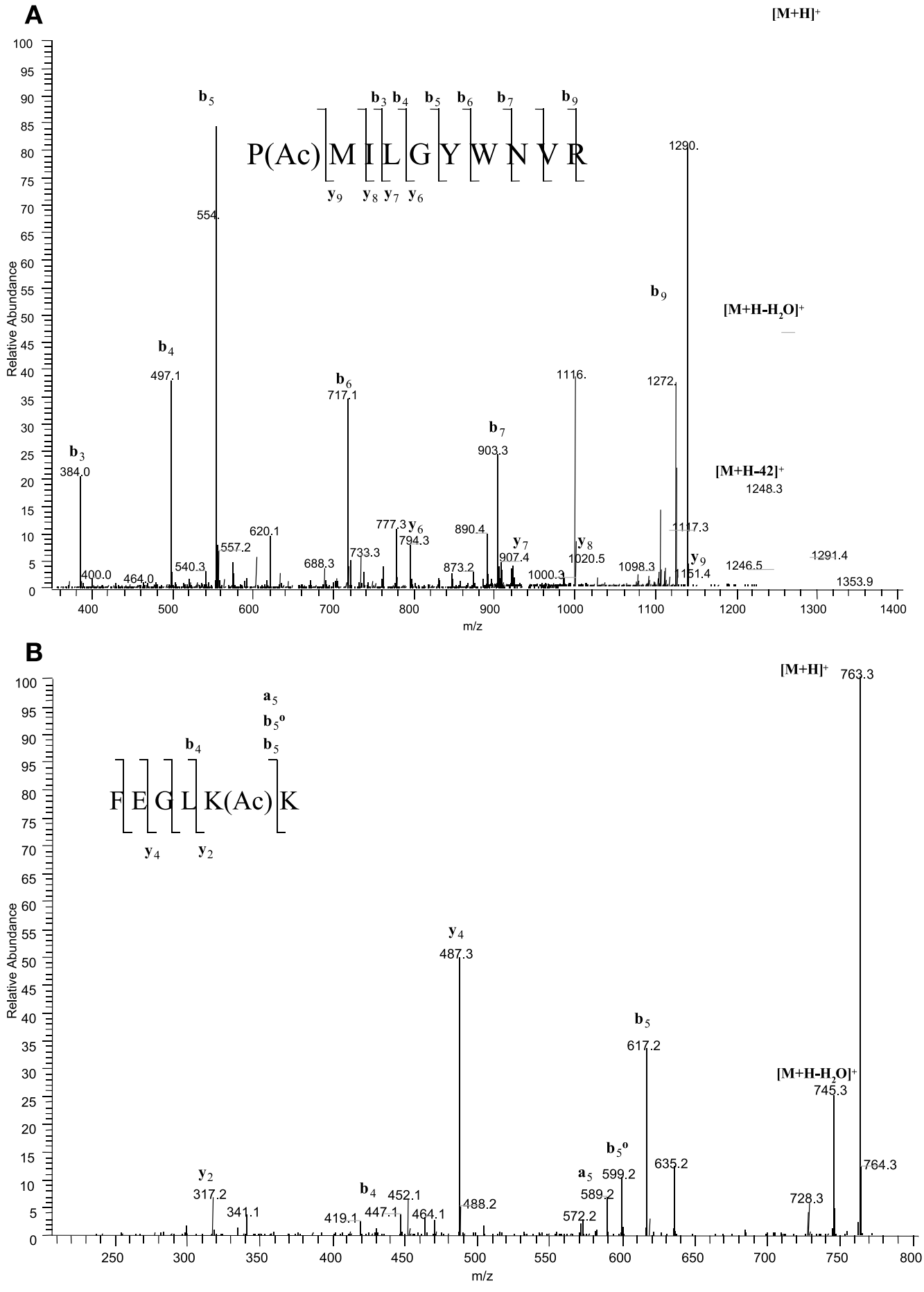
3. Results

The enzyme TAase was purified from liver microsome of the buffalo. The purified enzyme preparation had a molecular weight of 63 kDa. In order to establish the TAase-catalyzed acetylation of GST by DAMC as acetyl donor, the recombinant GST3-3 was used as a model protein substrate. GST3-3 was incubated for 30 min with DAMC and TAase under the conditions of the assay described in Section 2. The reaction mixture was subjected to SDS–PAGE in order to separate GST3-3 from the TAase. The subunit of GST3-3 considered for MS analysis was devoid of any contaminating proteins, which were removed by SDS–PAGE. The tryptic peptides were extracted from the gel pieces and the unfractionated tryptic digest was analyzed by MALDI-TOF MS. The predicted tryptic peptides were identified using Mascot Search Engine (www.matrixscience.com). The peptide maps so obtained covered 97% of the GST3-3 sequence. By overlaying MALDI peptide maps of modified and control GST, seven new peaks, corresponding to potentially acetylated peptides, were detected in the modified map. The mass values of each of them 42 kDa higher than the theoretical mass of native GST tryptic peptides were 1290.68 (T_{1-10}); 2107.15 (T_{50-67}); 1566.87 (T_{82-93}); 931.54 ($T_{122-128}$); 1721.85 ($T_{173-186}$); 763.33 ($T_{187-192}$) and 1203.77 ($T_{202-217}$), strongly suggesting acetylation. CID spectra obtained were of good quality. Acetylation at several points in the GST sequence was suggested by MALDI, but final confirmation was possible by the application of micro (μ)LC/MS/MS. Tryptic peptides originated from in-gel digestion of the modified GST were separated by microbore reversed phase liquid chromatography (RPLC), and peptides of interest were fragmented in an ion trap mass spectrometer in order to get structural information and to pinpoint amino acid modifications. CID spectra were obtained for seven peptides and two examples are shown in Fig. 2A and B. By examining the fragmentation pattern and by comparing experimental and predicted values for MS/MS ions, the identity of the seven acetylated GST tryptic peptides could be confirmed. The N-terminal proline and the six lysines, Lys-51, -82, -124, -181, -191 and -210, were found acetylated.

4. Discussion

Our perception of acetyl CoA-independent acetylation of proteins had its origin in our investigations on the mechanism

Fig. 2. A: MS/MS spectrum of peptide T_{1-10}; *m/z* 1290.5 [$(M+H)^+$, 100]. B: MS/MS spectrum of peptide $T_{187-192}$; *m/z* 763.3 [$(M+H)^+$, 100]. →


A
[M+H]+
P(Ac)MILGYWNVR
b5
b3 b4 b5 b6 b7 b9
y9 y8 y7 y6
554.
1290.
b9
[M+H-H2O]+
b4
497.1
b6
717.1
1116.
1272.
b7
903.3
b3
384.0
[M+H-42]+
1248.3
620.1
777.3 y6
794.3
890.4
y7
907.4
y8
1020.5
y9
1117.3
1151.4
1246.5
1291.4
1353.9
557.2
733.3
688.3
873.2
1000.3
1098.3
540.3
400.0
464.0
Relative Abundance
m/z
B
[M+H]+
763.3
a5
b5o
b5
b4
FEGLK(Ac)K
y4 y2
y4
487.3
b5
617.2
[M+H-H2O]+
745.3
b5o
635.2
764.3
y2
317.2
a5 599.2
589.2
728.3
b4 452.1
341.1
447.1
464.1
488.2
419.1
572.2
Relative Abundance
m/z

of biochemical action of DAMC. We had earlier demonstrated the remarkable inhibitory action of DAMC on several microsomal cytochrome *P*-450-linked mixed function oxidases (MFO) catalyzing aflatoxin B_1-epoxidation, dealkylation of alkylated resorufins [6,10] and toxicokinetics of benzene [11]. The irreversible inhibition of MFO mediated by DAMC was in contrast to the action of classical inhibitors of cytochrome *P*-450 [12–15] in that DAMC needed no prior oxidative metabolism. Also, the potent inhibition of cytochrome *P*-450-linked MFO by DAMC was nearly abolished by thiol blocking agents such as *p*-hydroxy mercuribenzoate (PHMB) and iodoacetamide [8]. Such observations strongly connoted the existence of a new enzyme in microsomes, termed DAMC:protein transacetylase (TAase) catalyzing the transfer of acetyl groups of DAMC to the apoprotein of cytochrome *P*-450 resulting in the mechanism-based inhibition of MFO. Further investigations revealed that two other enzyme proteins were possibly modified by TAase-catalyzed action of DAMC. Accordingly, the hyperbolic activation of NADPH cytochrome *c* reductase and the mechanism-based inhibition of GST were recorded [7,8]. The irreversible inhibition of cytosolic GST caused by incubation of DAMC with buffalo liver microsomes served as the basis of an elegant assay procedure for TAase [8] and with this assay preliminary characterization of TAase was carried out. TAase exhibited hyperbolic kinetics; K_m and V_{max} values obtained by varying DAMC or GST concentration highlighted the nature of TAase-catalyzed reaction as bimolecular. The formation of 7,8-dihydroxy-4-methyl coumarin (DHMC), a product of the TAase-catalyzed reaction (Fig. 1), was identified by us [8,10]. The convincing biochemical reasoning outlined above pointed out the existence of a new enzyme, TAase. Nevertheless, the hard-core evidence to this effect in terms of protein (GST) acetylation per se, amounting to the demonstration of the other product (acetylated GST) of the TAase-catalyzed reaction was essential. The realization of this objective necessitated in the first place the purification of TAase and the purified preparation of TAase was utilized to study the acetylation of GST by DAMC. Purified TAase from buffalo liver microsomes catalyzed the acetylation of GST3-3 by DAMC. The well-established mass spectrometric methods were used to identify the sites. N-terminal proline and six lysines in GST3-3 were found to be acetylated. The acetylation catalyzed by TAase was found clearly distinct from the acetylation of GST as a consequence of natural post-translational modification where N-terminal residue alone was modified.

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