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Acetoxy drug: protein transacetylase: A novel enzyme-mediating protein acetylation by polyphenolic peracetates*

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Abstract: The acetylation of proteins in biological systems is largely catalyzed by specific acetyl transferases utilizing acetyl CoA as the acetyl donor. The enzymatic acetylation of proteins independent of acetyl CoA was unknown until we discovered a unique membrane-bound enzyme in mammalian cells catalyzing the transfer of acetyl groups from polyphenolic peracetates (PAs) to certain enzyme proteins, resulting in the modulation of their catalytic activities. Since for the enzyme, acetyl derivatives of several classes of polyphenols such as coumarins, flavones, chromones, and xanthones were found to be acetyl donors, the enzyme was termed as acetoxy drug: protein transacetylase (TAase). TAase was found to be ubiquitously present in tissues of several animal species and a variety of animal cells. Liver microsomal cytochrome P-450 (CYP), NADPH-cytochrome c reductase and cytosolic glutathione *S*-transferase (GST) were found to be the targets for TAase-catalyzed acetylation by the model acetoxy drug 7,8-diacetoxy-4-methylcoumarin (DAMC). Accordingly, the catalytic activities of CYP-linked, mixed function oxidases (MFOs) and GST were irreversibly inhibited while the reductase was remarkably activated. In this report, we have reviewed the details concerning purification and characterization of TAase and the protein acetylation by DAMC. Quantitative structure–activity relationship (QSAR) studies concerning the specificities of various PAs to liver microsomal TAase and TAase-related biological effects have also been reviewed.

INTRODUCTION

Acetylation is the most common covalent modification of proteins encountered in living cells. The specific acetyl transferases catalyze the transfer of acetyl group which is bound by a high-energy bond to

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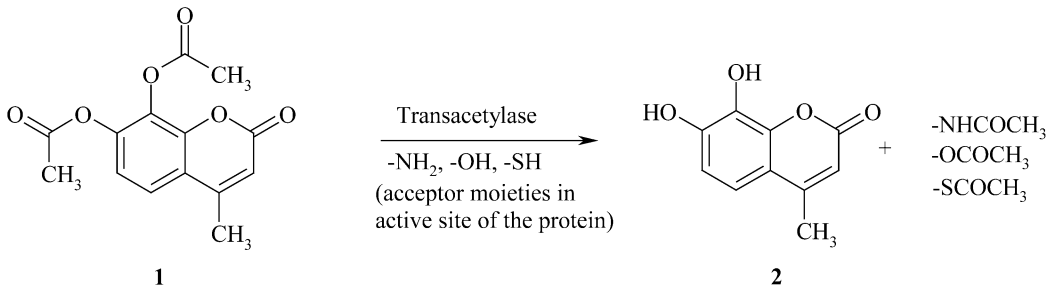
the free SH group of coenzyme A (CoA) to proteins. Acetyl CoA-dependent acetyl transferases play a major role in the acetylation of proteins [1]. Proteins are also nonenzymatically acetylated, the familiar example being the acetylation of cyclooxygenase by aspirin [2]. The perception of acetyl CoA-independent enzymatic acetylation of proteins had its origin in our investigations on the mechanism of biochemical action of 7,8-diacetoxy-4-methylcoumarin (**1**, DAMC). We had earlier demonstrated the remarkable inhibitory action of **1** on several liver microsomal cytochrome P-450-linked mixed function oxidases (MFOs) catalyzing aflatoxin B_1- (AFB_1) epoxidation, dealkylation of resorufin [3], and toxicokinetics of benzene [4]. The irreversible inhibition of MFO mediated by **1** was in contrast to the action of classical inhibitors of P-450 [5–8] in that **1** needed no prior oxidative metabolism. Also, the inhibition of MFO by **1** was nearly abolished by thiol blocking agents such as *p*-hydroxymercuribenzoate and iodoacetamide. 7,8-Dihydroxy-4-methylcoumarin (**2**, DHMC), the deacetylated product of **1** failed to elicit irreversible inhibition of liver microsome-catalyzed AFB_1-epoxidation (measured as DNA binding) [3]. These results connoted the existence in liver microsomes of a thiol enzyme, which could be responsible for the transfer of acetyl group of **1** to the apoprotein of P-450 causing irreversible inhibition of MFO; **1** was also found effective in causing the in vivo inhibition of AFB_1 binding to DNA in rats administered with AFB_1. Furthermore, the pretreatment of rats with **1** (unlike **2**) resulted in significant reduction in clastogenic response (measured by the incidence of micronuclei and apoptotic bodies formation) in rat lungs and bone marrow cells of rats injected with AFB_1 [9].

RESULTS AND DISCUSSION

TAase-catalyzed inhibition of cytosolic glutathione *S*-transferase by 7-8-diacetoxy-4-methylcoumarin

Further investigations revealed that the incubation of glutathione *S*-transferase (GST) with rat liver microsomes and **1** resulted in the mechanism-based inhibition of GST. Also, the inclusion of thiol blocking agents in the incubation mixture reversed the inhibition of GST and **2** failed to cause inhibition of GST. These results substantiated the role of TAase in the possible inhibition of GST by **1**. The irreversible inhibition of cytosolic GST caused by incubation of **1** with rat liver microsomes served as the basis of an elegant assay procedure (Fig. 1) for TAase [10], and, utilizing this assay procedure, preliminary characterization of TAase was carried out. TAase exhibited hyperbolic kinetics; K_m and V_{max} obtained by varying **1** or GST concentration highlighted the nature of TAase-catalyzed reaction as bimolecular. TAase was purified from buffalo liver, the purified enzyme was found to have approximately mol wt of 63 kDa.



1 **2**

Fig. 1 Liver microsomal TAase catalyzing the transfer of acetyl groups to $–NH_2$, –OH, –SH moieties of proteins.

Demonstration of TAase-catalyzed acetylation of GST 3-3 by 7-8-diacetoxy-4-methylcoumarin

The biochemical evidences mentioned above pointed out that the acetylation of proteins was possibly catalyzed by DAMC: protein transacetylase (TAase). In order to provide hard-core evidence to this, efforts were made to establish TAase-catalyzed protein acetylation by protein mass spectrometry. For this purpose, GST 3-3 (isoform of GST) was used as a model protein substrate, which was incubated for 30 min with **1** and purified buffalo liver TAase under the conditions of TAase assay procedure as described in our earlier report [10]. The modified GST was separated by SDS-PAGE, in gel digested with trypsin, and the tryptic digest was analyzed by MALDI TOFMS and LC/MS/MS. The N-terminal proline and six lysines: Lys-51, -82, -124, -181, -191, and -210 were found to be acetylated [11,12]. These findings confirm the phenomenon of enzymatic protein acetylation independent of acetyl CoA by a hitherto unknown enzyme (TAase) using a xenobiotic phenolic acetate such as **1** as the substrate.

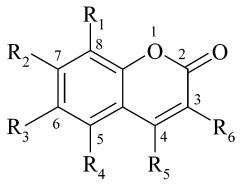
Quantitative structure–activity relationship (QSAR) studies with TAase

After having established that **1** is a good substrate for rat liver microsomal TAase, we then proceeded to establish the specificity of acetoxy-coumarin to rat liver microsomal TAase. For this purpose, coumarins **3–9** bearing one acetoxy group separately at C-3, C-4, C-5, C-6, or C-7 positions were synthesized and specificities to TAase were examined.

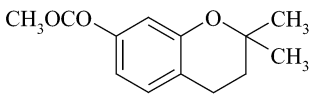
Negligible TAase activity was apparent with 3-acetoxycoumarin (**8**) as the substrate, while the substrate efficiency of other acetoxycoumarins were in the order: 7-acetoxy-4-methylcoumarin (**3**) > 6-acetoxy-4-methylcoumarin (**4**) > 5-acetoxy-4-methylcoumarin (**5**) = 4-acetoxycoumarin (**7**). In order to achieve a comparable level of TAase activity measured in terms of inhibition of GST [10], the required concentrations of 7-acetoxy-4-methylcoumarin (**3**), 6-acetoxy-4-methylcoumarin (**4**), 5-acetoxy-4-methylcoumarin (**5**), and 4-acetoxycoumarin (**7**) were in the order 1:2:4:4, respectively; **1** was found to elicit maximum level of GST inhibition, nearly twice that of 7-acetoxy-4-methylcoumarin (**3**) [13]. These findings convincingly demonstrated that a high degree of acetyl group transfer capability was conferred when the acetoxy group on the benzenoid ring of the coumarin system is in closer proximity to the oxygen heteroatom, i.e., when the acetoxy groups are at the C-7 and C-8 positions. We further observed that in **1**, the C-7 acetoxy group helps the C-8 acetoxy group to orient itself most favorably toward the oxygen heteroatom of the coumarin moiety as revealed by the optimized structures. Also, the relative orientation of acetoxy group toward the oxygen heteroatom was found to be maximum in case of **1** and the least in the case of 3-acetoxycoumarin (**8**) [13]. The investigations on the substrate specificity to TAase were extended to the peracetates **10**–**27** of other polyphenols, i.e., flavones, isoflavones, catechin, etc. with a view to establish the importance of pyran carbonyl group for the catalytic activity [14]. The absolute requirement of the carbonyl group in the pyran ring of the substrate for TAase activity was evident by the observation that TAase activity was hardly discernible when catechin pentaacetate (**12**) or 7-acetoxy-3, 4-dihydro-2, 2-dimethylbenzopyran (**10**) (both lacking carbonyl group) were used as the substrates [14].

Furthermore, the specificity of acetoxy-4-phenylcoumarins and acetoxy-4-phenyldihydrocoumarins was compared to that of acetoxy derivatives of 4- methylcoumarins. The addition of phenyl moiety to the pyranone moiety of the acetoxycoumarins caused drastic reduction in their specificity to TAase. Accordingly, 7-acetoxy-4-phenylcoumarin (**9**)/7-acetoxy-4-phenyldihydrocoumarin (**11**) yielded significantly less activity when used as the substrate for liver microsomal TAase as compared to the acetoxy derivatives of 4-methylcoumarins [15].

These results offered a plausible explanation for the finding that acetoxyflavones/isoflavones exhibited much less specificity to TAase when compared to the corresponding acetoxycoumarins in that the phenyl ring attached at C-2 or C-3 position of the pyran ring, respectively, was responsible for the inhibition of TAase activity [13,14].



3. R_2=OAc, R_5=CH_3, R_1=R_3=R_4=R_6=H
4. R_3=OAc, R_5=CH_3, R_1=R_2=R_4=R_6=H
5. R_4=OAc, R_5=CH_3, R_1=R_2=R_3=R_6=H
6. R_4=OAc, R_5=R_2=CH_3, R_1=R_3=R_6=H
7. R_5=OAc, R_1=R_2=R_3=R_4=R_6=H
8. R_6=OAc, R_1=R_2=R_3=R_4=R_5=H
9. R_2=OAc, R_5=Ph, R_1=R_3=R_4=R_6=H



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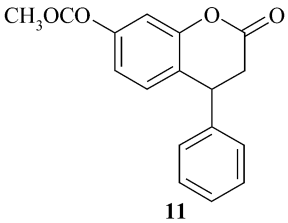

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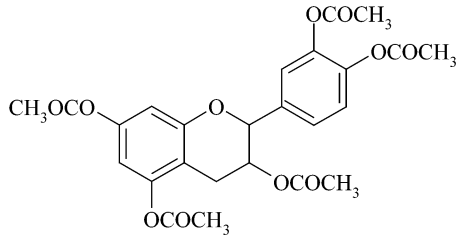

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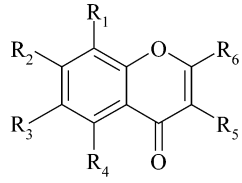

13. R_1=R_2=R_3=OAc, R_4=[3,4-$(OAc)_2$]Ph, R_5=H
14. R_2=R_5=OAc, R_6=(4-OAc)Ph, R_1=R_3=R_4=H
15. R_2=OAc, R_5=(2-OAc)Ph, R_1=R_3=R_4=R_6=H
16. R_1=CH_3, R_2=OCH_3, R_4=OAc, R_5=(4-OCH_3)Ph, R_3=R_6=H
17. R_1=R_5=OCH_3, R_4=OAc, R_6=[3,4,5-$(OCH_3)_3$]Ph, R_2=R_3=H
18. R_1=R_2=R_3=OCH_3, R_4=OAc, R_6=[3,5- $(OAc)_2$, 4-OCH_3]Ph, R_5=H
19. R_2=OCH_3, R_4=OAc, R_6=(4-OAc)Ph, R_1=R_3=R_5=H
20. R_2=R_5=OCH_3, R_3=R_4=OAc, R_6=(4-OCH_3)Ph, R_1=H
21. R_1=R_4=OAc, R_2=R_3=OCH_3, R_6=Ph, R_1=R_5=H
22. R_1=R_4=OAc, R_2=OCH_3, R_5=OEt, R_6=[3,4,5-$(OCH_3)_3$]Ph, R_3=H
23. R_1=R_2=R_4=OAc, R_5=OCH_3, R_6=Ph,R_1= R_3=H
24. R_1=R_2=R_4=OAc, R_5=Ph, R_3=R_6=H
25. R_1=R_2=OAc, R_5=Ph, R_3=R_4=R_6=H
26. R_2=R_4=R_5=OAc, R_6=[3,4-$(OAc)_2$]Ph, R_3=H
27. R_2=R_5=OCH_3, R_6=(3-OAc, 4-OCH_3)Ph,R_1=R_3=R_4=H

TAase-RELATED BIOLOGICAL EFFECTS

The TAase-mediated acetylation of functional proteins utilizing polyphenolic peracetates (PAs) as the acetyl donors results in the expression-altered physiological effects. The physiological implications of TAase-related biological effects, which may be exploited for the development of candidate drugs, are described here.

TAase as a new player in drug metabolism

As described earlier, PAs irreversibly inhibit cytochrome P-450-linked MFO through the action of TAase. TAase can thus be considered to play a vital role in modulating the metabolism of xenobiotics utilizing PA as the acetyl donor. Polyphenols are known to modulate cytochrome P-450-linked MFO [16]. Acetylated polyphenols can be superior in their ability to modulate MFO compared to polyphenols. This postulate was found to be true when we compared the prevention of genotoxicity of AFB_1 in bone marrow cells by quercetin and quercetin pentaacetate, the latter was found to be more effective in preventing the genotoxicity [17].

TAase-catalyzed irreversible activation of NADPH cytochrome c reductase by PAs

Efforts were made to examine whether NADPH cytochrome c reductase (a component of the microsomal electron transport system) is possibly a target for the action of PA-mediated action of microsomal TAase. For this purpose, liver microsomes were preincubated with **1**, followed by the assay of reductase. The preincubation of liver microsomes with **1** resulted in a time-dependent activation of the reductase. The catalytic activity of reductase was enhanced nearly 600 % by 25 μM concentration of **1** after 10 min of preincubation [18]. The action of **1** on the liver microsomal reductase resulted in the enhancement of V_{max} while K_m remained constant. A plot of $1/V_{max}$ against concentration of **1** resulted in a nonlinear, but a rectangular hyperbola indicative of hyperbolic activation of the reductase; **1** also proved to be effective in significantly enhancing the activity of reductase in vivo. The hydrolysed product of **1**, i.e., **2** failed to irreversibly activate the reductase. The activation effect of **1** upon the reductase was greatly inhibited by thiol blocking agent *p*-hydroxymercuribenzoate. These observations confirmed the role of microsomal TAase in catalyzing the activation of reductase by **1** [18].

It was thought interesting to examine whether nitric oxide synthase (NOS), which bears a domain of NADPH cytochrome c reductase, could similarly be activated leading to enhanced levels of NO. In order to investigate this proposition, we chose human platelets as the suitable experimental system. Human platelets were found to have considerable TAase activity. TAase-catalyzed activation of platelet reductase by PAs was also investigated, platelet NOS was indeed found to be activated by incubation with PAs in tune with their specificities to TAase [15]. The search for acetoxy drugs that can mediate TAase-catalyzed activation of NOS would assume importance as these agents can enhance intracellular NO levels.

CONCLUSIONS

The investigations carried out in our laboratories concentrated on the role of a novel enzyme TAase catalyzing the transfer of acetyl group from a variety of PAs to certain functional proteins. TAase was purified and characterized from buffalo liver. The acetylation of GST3-3 isoform by **1** mediated by buffalo liver TAase was established by mass spectrometry. The detailed QSAR studies on the acetylated polyphenols revealed the structural features governing the specificity to TAase. TAase-catalyzed modification of CYP-linked MFO and NOS by PAs could be of considerable pharmacological significance.

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