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Mechanism of Biochemical Action of Substituted 4-Methylbenzopyran-2-ones. Part 4: Hyperbolic Activation of Rat Liver Microsomal NADPH-Cytochrome C Reductase by the Novel Acetylator 7,8-Diacetoxy-4-methylcoumarin

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Abstract—The effect of 7,8-diacetoxy-4-methylcoumarin (DAMC) has been studied on hepatic NADPH cytochrome C reductase—an enzyme participating in the microsomal electron transport. The preincubation of liver microsomes with DAMC resulted in a time-dependent activation of NADPH cytochrome C reductase. The catalytic activity of the enzyme enhanced nearly 600% by 25 μM concentration of DAMC after 10 min of preincubation. The action of DAMC on the reductase resulted in enhanced v_{max} while K_m remained constant. A plot of $1/v_{max}$ as a function of DAMC concentration resulted in a non-linear, but rectangular hyperbola indicative of hyperbolic activation. DAMC was also proved to be effective in significantly enhancing the activity of NADPH cytochrome C reductase in vivo. 7,8-Dihydroxy-4-methylcoumarin (DHMC), the deacetylated product of DAMC failed to irreversibly activate the enzyme. The activation effect of DAMC upon the enzyme was abolished by *p*-hydroxymercury benzoate. The role of a transacetylase in transferring the acetyl group of DAMC to the amino acid(s) of the active site of NADPH cytochrome C reductase causing irreversible enzyme activation is enunciated.

Introduction

Substituted 4-methylcoumarins are an important class of naturally occurring compounds. Though these are known to possess useful properties, such as choleretic[1] analgesic,[2] antispermatogenic[3] and diuretic,[4] much attention has not been focussed on the mechanism of action of this class of compounds. For this reason, we undertook investigations to unravel their biological effects keeping in view the mechanism underlining their unique mode of action. Our earlier reports[5] highlighted the ability of substituted dioxygenated 4-methylcoumarins to inhibit effectively the cytochrome P-450-linked mixed function oxidase. 7,8-Diacetoxy-4-methylcoumarin (DAMC) was found to cause mechanism based inhibition of liver microsome-catalysed aflatoxin B_1 (AFB_1)–DNA binding as well as of P-4501A and P-4501B activities[6] possibly due to acetylation of P-450 apoprotein catalysed by microsomal transacetylase (Fig. 1). It was thought interesting to examine the outcome of the action of DAMC on another enzyme participating in hepatic microsomal electron transport, namely NADPH cytochrome C-reductase. The results of the study concluded that DAMC, contrary to its inhibitory action on hepatic P450 caused kinetically discernible hyperbolic activation of liver microsomal NADPH cytochrome C reductase.

Materials and Methods

Chemicals

NADPH and cytochrome C were purchased from Sigma Chemical Co., St. Louis, MO, USA. Sodium *p*-hydroxymercury benzoate (PHMB) was procured from E. Merck (Germany). Other chemicals used were of AR grade and were procured from local suppliers. 7,8-Diacetoxy-4-methylcoumarin (DAMC) and 7,8-dihydroxy-4-methylcoumarin (DHMC) were synthesized in our Laboratory by the well known Pechmann condensation.[7]

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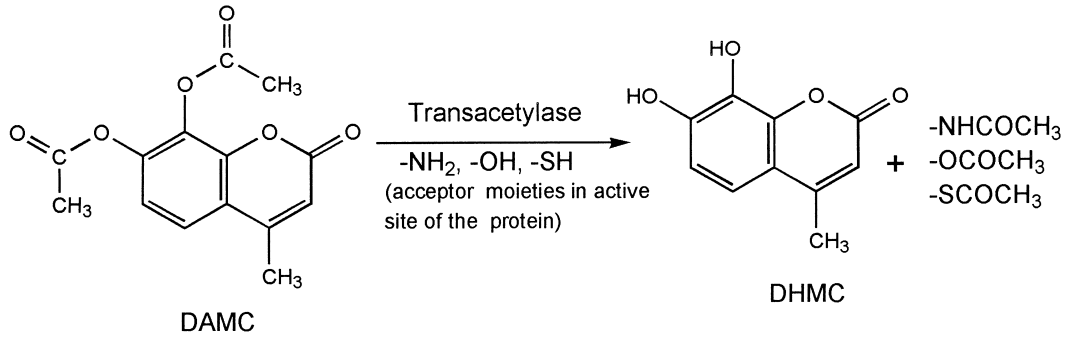

Figure 1. Liver microsomal transacetylase catalysing the transfer of acetyl group to -NH_2, -OH, -SH of protein.

Animals

Male albino rats of wistar strain weighing around 190–200 g, fed on rat chow supplied by Hindustan Lever Ltd., Mumbai (India) were used.

Preparation of liver microsomes

Male rats were sacrificed, liver excised and microsomes prepared as described earlier.[5,8]

Preparation of DAMC -treated liver microsomes

Rats were injected DAMC i.p. (300 mg/kg body weight) in 0.2 ml DMSO, followed 1 h later by administration of another dose of DAMC. The animals were sacrificed 2 h after the second injection, liver excised, homogenised and microsomes prepared as mentioned above.

Assay of NADPH cytochrome C reductase

The method of Masters et al.[9] was followed, the assay mixture consisted of 0.05 M phosphate buffer (pH 7.7), 0.1 mM EDTA, liver microsomes (25 μg protein) and cytochrome C (concentration of cytochrome C was varied according to nature of kinetic studies). The reaction was started by the addition of 1.0 mM NADPH in a total volume of 1.0 ml. The progress of the reaction was followed by measuring the absorption at 550 nm using Backman UV–VIS spectrophotometer Model DU64.

Kinetics of time dependent activation of NADPH cytochrome C reductase by DAMC

Rat liver microsomes (25 μg protein) were mixed with a fixed concentration of DAMC (2–10 μM added in 0.2 mL DMSO) in 0.05 M phosphate buffer (pH 7.7) and water was added to make 0.8 mL volume. The contents (scaled up as per requirement) were preincubated at 37 °C in a shaking water bath. The aliquots (0.5 mL portion) were removed periodically into another set of tubes containing 0.1 mM EDTA, 36 μM cytochrome C and 1 mM NADPH in a total volume of 0.1 ml. The progress of the reaction was followed by monitoring absorption at 550 nm as described above.

Kinetics of activation of NADPH cytochrome C reductase by DAMC

The rat liver microsomes were separately incubated for 10 min with different concentrations of DAMC (0–25 μM) as described earlier and aliquots were removed for the assay of NADPH cytochrome C reductase at varying substrate (cytochrome C) concentrations (18–90 μM). The reaction was started by the addition of 1.0 mM NADPH in a total volume of 1.0 ml and the progress of the reaction was followed as described above. In experiments where DHMC was used, the procedure described here was followed replacing DAMC by DHMC.

Effect of PHMB on DAMC mediated cytochrome C reductase

PHMB was added during the preincubation of microsomes with DAMC at a concentration of 10^{-2} M and the procedure described above was followed.

Kinetics of DAMC-treated rat liver microsomal NADPH cytochrome C reductase

The DAMC-treated liver microsomes (prepared as described earlier) were assayed for kinetic measurements of the reductase activity according to the procedure described earlier.

Results

The enzymatic reduction of cytochrome C catalysed by liver microsomes in the presence of NADPH was followed by increase in absorption at 550 nm. The conditions of the assay were controlled to assure that the true initial velocity of the reaction was linear with respect to the enzyme concentration. Liver microsomes were preincubated with a known concentration of DAMC for different periods of time, followed by the determination of the initial rate of the activity of NADPH cytochrome C reductase. The percent activation of the reductase was plotted against time of preincubation of microsomes with DAMC. For kinetic studies, the assays were conducted in duplicate and the results represent average

with variation less than 5%. The results described in Figure 2 clearly indicate the enzyme activation due to DAMC in a concentration dependent manner. Liver microsomal NADPH cytochrome C reductase was activated nearly 200% by 10 μM and 600% by 25 μM concentration of DAMC after 10 min of preincubation, respectively (Figs 2 and 3).

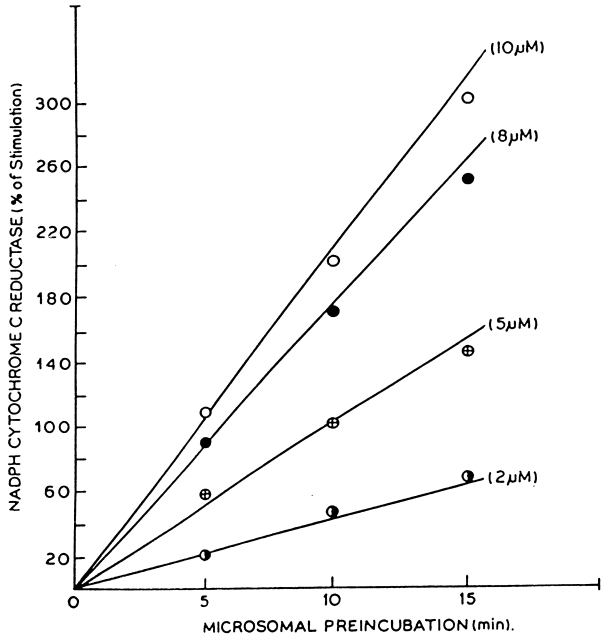

Figure 2. Liver microsomes were separately preincubated with DAMC (2 to 10 μM) for different periods of time, followed by the assay of NADPH cytochrome C reductase. Each point represents measurements made in duplicate with variation less than 5%.

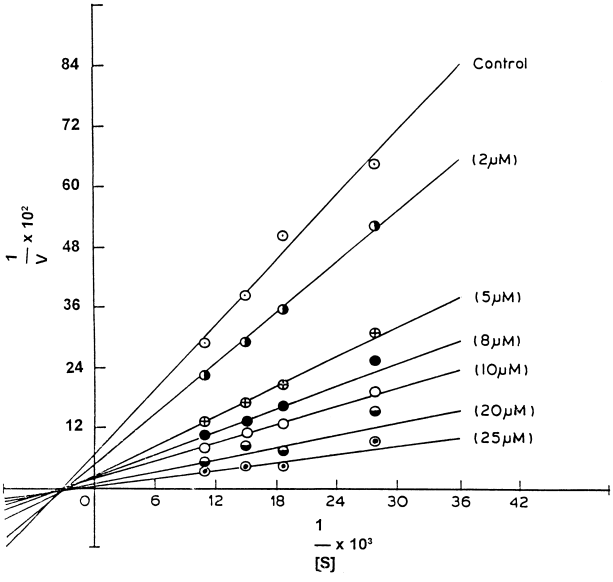

Figure 3. Liver microsomes were separately preincubated with DAMC (0 to 25 μM) for 10 min., followed by the assay of NADPH cytochrome C reductase varying the substrate concentration (cytochrome C) in order to obtain the double reciprocal plot. The details are described under 'Materials and Methods'.

v: initial rate expressed as concentration of cytochrome C(μM) reduced per min. S: substrate concentration (μM).

Each point represents the mean of two measurements with variation $< 5\%$.

Liver microsomes were preincubated in multiple sets with different concentrations of DAMC (2–25 μM) separately for 10 min. At the end of preincubation, the components of the reaction mixture were added to the tubes as described under 'Materials and Methods', except for cytochrome C which was added in varying concentrations. The reaction was started with the addition of NADPH and the initial rate determined. The data were plotted (Fig. 3) to obtain double reciprocal plot (1/[v] against 1/[s]). The results clearly demonstrate activation of the reductase catalytic activity by DAMC in DAMC-concentration dependent manner (Fig. 3). The kinetics of activation of NADPH cytochrome C reductase by DAMC revealed enhanced v_{max}, while K_m remained constant (Fig. 3). The nature of the enzyme activation by DAMC was examined by plotting the reciprocal of v_{max} (as described for Figure 3) as a function of the concentration of DAMC. The result (Fig. 4) clearly indicates that DAMC causes hyperbolic activation of NADPH cytochrome C reductase.

The data tabulated in Table 1 demonstrate the effect of PHMB on the DAMC-mediated activation of liver microsomal NADPH cytochrome C reductase. Addition of PHMB to the reaction mixture completely abolished the marked activation of NADPH cytochrome C reductase brought about by DAMC (Table 1). It is pertinent to note that PHMB alone at the concentration used unaltered the catalytic activity of the reductase (Table 1). Efforts were made to examine whether DAMC can indeed activate NADPH cytochrome C reductase in vivo. For this purpose, rats were administered i.p. DAMC and liver microsomes prepared as mentioned under 'Materials and Methods'. The

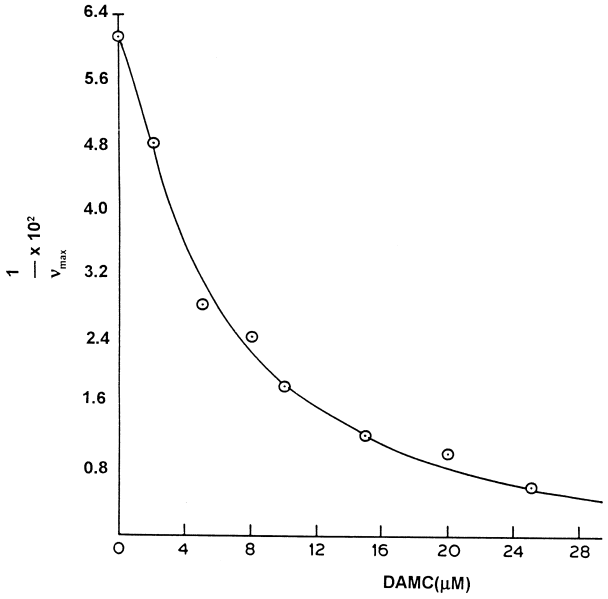

Figure 4. Liver microsomes were separately preincubated with DAMC (0 to 25 μM) followed by the kinetic assay of NADPH cytochrome C reductase as described in Figure 3.

Table 1 Influence of PHMB on time dependent activation of NADPH cytochrome C reductase by DAMC

Preincubation (min)	NADPH cytochrome C reduced (μmoles/min/ml×10^{-5})			
	Control	DAMC	DAMC + PHMB	PHMB
5	152.4	300.6	162.0	171.6
10	143.1	429.3	171.6	147.9
15	162.0	572.4	157.2	166.8

Concentration of DAMC = 10 μM
Concentration of PHMB = 10^{-2} M
Values represent average of two assays.

Table 2 Influence of DHMC on rat liver microsomal NADPH cytochrome C reductase

Preincubation (min)	NADPH cytochrome C reduced (μmoles/min/ml×10^{-5})			
	A	B	C	D
5	238.5	429.3	572.4	620.1
10	238.5	424.5	581.7	629.4
15	233.7	429.3	581.7	620.1

Values represent average of two assays.
A, B, C and D represent concentration of DHMC (μM): 2,5,8 and 10, respectively.

kinetics of the reductases of normal and DAMC-treated microsomes clearly indicated irreversible activation of reductase by DAMC (Fig. 5).

The results described in Figure 5 reveal higher v_{max} for DAMC-treated microsomes as compared to microsomes from untreated rats, thereby confirming the competency of DAMC to irreversibly activate NADPH cytochrome C reductase in vivo. It was thought interesting to examine whether DHMC, the deacetylated product of DAMC has the ability to irreversibly activate rat liver microsome NADPH cytochrome C reductase. The results embodied in Table 2 reveal that DHMC, unlike DAMC upon preincubation with liver microsomes failed to cause time dependent activation of catalytic activity of the NADPH cytochrome C reductase.

Discussion

NADPH cytochrome C reductase of liver microsomes is a flavoprotein which has been purified and characterized.[9] This enzyme in its native milieu serves as a member of the microsomal electron transport chain directed towards hydroxylations. The enzyme is specific for NADPH but relatively nonspecific to cytochrome C, cytochrome P-450, or ferricyanide. The mechanism of electron transfer catalysed by NADPH cytochrome C reductase is well documented.[10] During the course of our earlier investigations on the search for compounds that inhibit aflatoxin B_1 binding to DNA, we observed that several dioxygenated 4-methylcoumarins, especially the diacetoxycoumarins were very efficient in inhibiting liver microsome-catalysed AFB_1 epoxidation.[5] Detailed study on the mechanism of inhibition of AFB_1–DNA binding by acetoxy-4-methylcoumarins, especially the 7,8-diacetoxy-4-methylcoumarin (DAMC) strongly suggested the mechanism-based inhibition of microsomal cytochrome P-450 [6] due to acetylation of aminoacids (possibly lysine) at the active centre of P-450 similar to the action of chloramphenicol.[11] We postulated the role of microsomal transacetylase (a thiol containing protein) in the transfer of acetyl moiety to the P-450 [6] (Fig. 1). These investigations on the action of DAMC were extended to another member of the P-450 pathway, namely NADPH cytochrome C reductase. The data embodied in this report convincingly describe DAMC as an irreversible activator of the reductase, in contrast to the inhibitory action on P-450. The kinetics of activation of NADPH cytochrome C reductase by DAMC obtained by plotting $1/v_{max}$ as a function of the activator (DAMC) concentration yielded nonlinear, but indeed a rectangular hyperbola (Fig. 4) and for this reason, the kinetic behaviour is termed as hyperbolic activation.[12] It is pertinent to point out that very few examples have been reported for hyperbolic activation of enzymes[12] and the action of DAMC on NADPH cytochrome C reductase reported here contributes a rare example. Hepatic microsomal NADPH cytochrome C reductase is known to be induced by agents such as phenobarbital.[13] But the irreversible activation due to the chemical modification of the enzyme as reported here is rather unknown. The ineffectiveness of DHMC (the deacetylated product of DAMC) to irreversibly activate the reductase strongly points out the role of acetylation of protein in the modification of catalytic activity. The action of PHMB (Table 1) is an indicator of thiol specific nature of the transacetylase catalysing the transfer of acetyl moiety from DAMC to the apoprotein of NADPH cytochrome C reductase. Further studies on DAMC-mediated modification of protein may prove useful in suitably altering the macromolecular receptors leading to the expression of desired pharmacological activity.

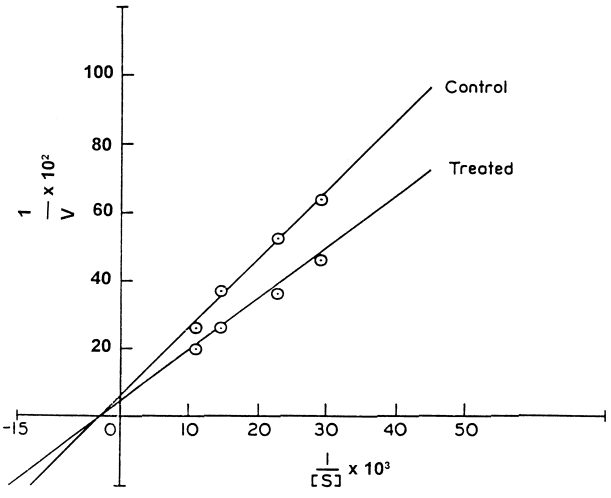

Figure 5. Liver microsomes from control and DAMC treated rats were assayed for NADPH cytochrome C reductase in order to compare the kinetic constants. The details are described under 'Materials and Methods'.

Acknowledgements

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References and Notes

1. Takeda, S.; Aburada, M. *J. Pharmacobio-Dyn.* **1981**, *4*, 724 (*Chem. Abstr. 1981*, *95*, 215075).
2. Yang, C. H.; Chaing, C.; Liu, K.; Perg, S.; Wang, R. *Yao Huseh Tung Pao* **1980**, *15*, 48 (*Chem. Abstr.* **1981**, *95*, 161758).
3. Tyagi, A.; Dixit, V. P.; Joshi, B. C. *Naturwissenschaften* **1980**, *67*, 104.
4. Deana, A. A.; Stokker, G. E.; Schultz, E. M.; Smith, R. L.; Cragoe, E. J.; Russo, H. F.; Watson, L. S. *J. Med. Chem.* **1983**, *26*, 580.
5. Raj, H. G.; Gupta, S.; Biswas, G.; Singh, S.; Singh, A.; Jha, A.; Bisht, K. S.; Sharma, S. K.; Jain, S. C.; Parmar, V. S. *Bioorg. Med. Chem.* **1996**, *4*, 2225.
6. Raj, H. G.; Parmar, V. S.; Jain, S. C.; Goel, S.; Singh, A.; Gupta, K.; Rohil, V.; Tyagi, Y. K.; Jha, H. N.; Olsen, C. E.; Wengel, J. *Bioorg. Med. Chem.* **1998**, *6*, 1895.
7. Parmar, V. S.; Bisht, K. S.; Jain, R.; Singh, S.; Sharma, S. K.; Gupta, S.; Malhotra, S.; Tyagi, O. D.; Vardhan, A.; Pati, H. N.; Berghe, D. V.; Vlietinck, A. J. *Indian J. Chem.* **1996**, *35B*, 220.
8. Allameh, A.; Saxena, M.; Raj, H. G. *Cancer Lett.* **1988**, *40*, 49.
9. Masters, B. S. S.; William, S. C. H.; Kamin, H. *Methods Enzymol.* **1967**, *10*, 565.
10. Masters, B. S. S.; Billimonia, M.; Kamin, H.; Gibson, Q. H. *J. Biol. Chem.* **1965**, *240*, 408.
11. Halpert, J. R. *Biochem. Pharmacol.* **1981**, *30*, 875.
12. *Fundamentals of Enzyme-Kinetics*; A. Cornish-Bowden, Ed. Portland Press Ltd: London, pp. 115–116.
13. Orrenius, S.; Ernster, L. *Biochem. Biophys. Res. Commun.* **1964**, *16*, 60.