行政院國家科學委員會專題研究計畫成果報告

生物性亞硫酸基轉移機制：

核甘酸與亞硫酸基轉移酵素之結合對於其型態變化與與催化反應之影響

MECHANISM OF SULFURYL GROUP TRANSFER IN BIOLOGICAL SYSTEM: HOW BINDING OF NUCLEOTIDE AFFECT CONFORMATION AND CATALYSIS OF PHENOL SULFOTRANSFERASE?

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中文摘要：

生物重要訊息之分辨與傳遞可質藉由硫酸化來達成，如：荷爾蒙之調節，解毒作用，及神經傳導等。由亞硫酸基轉移反應進行調控的物質有蛋白質、醣蛋白、醣類、荷爾蒙、神經傳導物質及許多外來物質，如藥物及致癌物等。生物質之硫酸化皆由亞硫酸基轉移酵素酵素催化，這一類酵素都 3'-Phospho Adenosine 5'-Phosphosulfate (PAPS) 或 3'-Phospho Adenosine 5'-Phosphate (PAP) 等核甘酸當作受質或輔因子。在已發表過的文獻中，尚未有亞硫酸基轉移酵素應用其它種核甘酸的報告，然而我們最近卻發現有其它種核甘酸可以用來取代 PAP 或 PAPS。有數種核甘酸不但可以與亞硫酸基轉移酵素酵素結合，還可以參與硫酸基轉移反應。本計畫則是想要了解其它種核甘酸與亞硫酸基轉移酵素酵素結合後，對於蛋白質形態上的變化及催化反應之影響，從而研究亞硫酸基轉移之反應機制。本計畫探討酵素催化不同種核甘酸之動力學，然後藉此挑選出適當的數種核甘酸做深入結構與功能關係之研究。

關鍵詞：硫酸化；核甘酸；亞硫酸基轉移酵素酵素；酵素反應機制。

Abstract

Nucleotides are required for the reaction of sulfuryl group transfer in biological system. Adenosine 3',5'-bisphosphate (PAP) or 3'-phospho adenosine 5'-phosphosulfate (PAPS) are cofactor or cosubstrate for sulfotransferase. We found that a variety of other nucleotides tightly bind to phenol sulfotransferase (PST) and three of them, adenosine 5'-monophosphate (AMP), adenosine 2',5'-bisphosphate (2',5'-PAP) and 2':3'-cyclic PAP, also involve in the sulfuryl group transfer reaction catalyzed by this enzyme. This is the first time that PST was shown to use nucleotides other than PAP to catalyze the reaction of sulfuryl group transfer. The binding of nucleotides with PST is rather non-selective. We have determined the dissociation constants of a variety of nucleotides and examined their potential as cofactors or cosubstrates for phenol sulfotransferase. HPLC and ^{31}P NMR are used to determine the activity of PST and identify the sulfated nucleotides. K_m and V_{max} of PST are determined when these nucleotides are used as cofactors or cosubstrates for sulfuryl group transfer. Values of V_{max}s for both the transfer and reverse physiological reactions remain relatively constant with all the different nucleotides tested. K_ms of PAP and AMP are in the μM and mM range, respectively, which reflect the physiological concentration of these two nucleotides in the cell.

Keywords: Sulfation; Nucleotide; Sulfotransferase; enzyme mechanism;

3'-Phospho Adenosine 5'-Phosphate (PAP); 3'-Phospho Adenosine 5'-Phosphosulfate (PAPS).

緣由與目的

Sulfation in a biological system has been known for a long time (Williams, 1947; 1959). Compounds which involve sulfation and hydrolysis of sulfate esters include drugs, carcinogens, other xenobiotics (Jakoby and Ziegler, 1990; Mulder, 1990), hormones (Pasqualini et al., 1992), bile acids (Roy, 1960), neurotransmitters (Kuchel et al., 1986), glycoproteins, glycosaminoglycans and saccharides (Roche et al., 1991). Unlike the better known phosphorylation reaction (Frey, 1992), the mechanism of enzymatic sulfuryl group transfer has yet to be understood (Duffel and Jakoby, 1987; Mulder and Jakoby, 1990).

A sulfated nucleotide, 3'-phospho adenosine 5'-phosphosulfate (PAPS) is required for the physiological sulfation catalyzed by sulfotransferases (EC 2.8.2) which are responsible for the catalyzation of sulfuryl group transfer reaction (Jakoby, 1990) as shown in equation 1. A two-step sulfuryl group transfer can be carried out by sulfotransferase with the catalytic amount of PAP as shown in Reaction 2 (Duffel and Jakoby, 1981). Phenol sulfotrasnferase catalyzed both reactions (Yang et al., 1996) with the activity in the physiological direction (Reaction 1) almost 10-fold lower than that in the transfer reaction (Reaction 2). Binding of PAP (Yang et al., 1998) and oxidation/reduction of cysteine (Marshall et al., 1997) which in turn affect the PAP binding may be the reason for the rate difference of the two reactions.

$$PAPS + HO\text{-}R \rightarrow PAP + R\text{-}OSO_3^{--} \quad (1)$$

$$R\text{-}OSO_3^{-} + R'\text{-}OH \rightarrow R\text{-}OH + R'\text{-}OSO_3^{-} \quad (2)$$

Sulfation of nucleotides other than PAP catalyzed by sulfotransferase has not been reported before. A recently published procedure (Lin and Yang, 1998) for the measurement of trace amount of PAP was found to be very useful for the initial screening of other potential cofactors for sulfuryl group transfer as Reaction 2. Following the initial screening, we are able to confirm, by HPLC and NMR, that three other adenine nucleotides can also be the cofactors or substrates of phenol sulfotransferase. We have found that even ribose and adenine, two major parts of the adenosine nucleotides, tightly bound to phenol sulfotransferase separately. Several nucleotides, adenosine 5'-phosphate, adenosine 2', 3'-cyclic phosphate 5'-phosphate, and adenosine 2', 5'-bisphosphate, were shown the first time to be sulfated at 5'-phopho position by a PST catalyzed reaction.

結果與討論

Two distinct dissociation constants for nucleotides and phenol sulfotransferase were determined and shown in Table 1. Phosphates at 2', 3' or 5' position (compare PAP, 2', 5'-PAP, AMP, ADP, ATP and adenosine) of the nucleotides do not affect their binding with enzyme (both K_{d1} and K_{d2} do not alter significantly). Removal of adenine base significantly affect the second dissociation constant as shown in the lower section of Table 1 (D-ribose and D-ribose 5'-phosphate). Only part of the nucleotide, adenine, D-ribose or D-ribose 5'-phosphat alone, is enough to bind PST tightly as indicated by the dissociation constants listed in the lower section of Table 1.

All the compounds listed in Table 1 were tested as cofactors for sulfuryl group

transfer between pNPS and β-naphthol as described in Materials and Methods. Those tested positive were listed in Table 2 and their K_m and V_{max} were determined. Unlike the K_ds of various nucleotides listed in Table 1, K_ms of the four nucleotides listed in Table 2 varied up to 10,000 fold. V_{max}s remain relatively constant with different nucleotides as cofactor.

Reactions of the transfer reaction indicate that nucleotides listed in Table 2 must be sulfated. The sulfated nucleotides were identified by HPLC and ^{31}P NMR as shown in Figures 1 and 2 in the absence of β-naphthaol as sulfuryl group acceptor. The elution times shown in Figure 1 is as expected in the condition described in Materials and Methods that less charged compounds move faster (less retention time). Thus, a slower moving compound appeared after the nucleotide was incubated with pNPS and PST as shown in Figure 1. Commercial available compounds, PAP, PAPS, 2',5'-PAP, AMP, 2':3'cyclic,5'-PAP were used as marker to identify the peaks appeared in Figure 1 (data not shown). Sulfation of adenine nucleotides at 5'-phospho position is clearly identified by ^{31}P NMR spectra at –10 ppm as shown in Figure 2. Progress of nucleotides sulfation also observed the decrease of chemical shift at 4.2 ppm for 5'-phosphate accompanied by the increase of chemical shift at –10 ppm for 5'-phosphosulfate (data not shown).

K_m and V_{max} of the sulfation of nucleotides were also determined and listed in Table 2. Similar to that of nucleotides as cofactor for the transfer reaction, V_{max}s remain to be relatively stable while K_m varied up to more than 1000 fold. The lack of a phospho group in adenine nucleotide increase K_m significantly for both the transfer and reverse physiological reactions.

Interaction of nucleotides with sulfotrasnferase is an important phenomena for the sutdy of this family of enzymes. AMP-, ATP-, and PAP-agarose have been used successfully as affinity chromatography for the purification of sulfotransferase (Sekura et al., 1981; Chen, 1981). Rescently, ADP-, ATP- and 2',5'-PAP-agarose were used for the analysis of flavonao 3-sulfotransferase and its mutants (Marsolais et al, 1999). However, binding of different nucleotides with sulfotransferase has not been previously characterized extensively. Dissociation constants listed in Table 1 indicate that even D-ribose or adenosine, part of the nucleotide, binds to phenol sulfotransferase tightly at the first binding sites. Several other nucleotides, such as uridine 5'-monophosphate and guanosine 5'-monophosphate, also tightly bind to phenol sulfotransferase at similar dissociation constants (data not shown). These observations explain why different nucleotides can be used as functional part of affinity chromatography for the purification of sulfotransferase. We concluded that phosphate at 2', 3' or 5' position of the adenine nucleotide is not important for the binding with phenol sulfotrnasferase. Both the ribose and the base (adenine or other base) may bind to the enzyme independently. Adenine is especially important for the nucleotide to bind to the second binding site of phenol sulfotransferase. However, efficiency of sulfation of nucleotides is dependent on the presence of adenine and 3'-phosphate.

The substrate spectrum of PST is much wider than as expected previously on the phenol part (Athena et. al.). Our data shows that this also applies to PST specificity of the nucleotide part. Two types of activated sulfate, APS and PAPS,

are used for PAPS synthase and sulfotransferase, respectivley. It has not been reported that phenol sulfotrasferase utilizes nucleotides other than PAP and PAPS. Sulfation of nucleotides catalyzed by PST is clearly demostrated by the results of HPLC and 31P NMR analysis (Figs. 1 and 2). PST appears to be very useful for the analysis of nucleotides. The procedure developed by Lin and Yang (1998) is a sensitive method for determination the concentration of PAP and for the identification of novel cofactor of PST.

While Vmax of the transfer of sulfuryl group between two phenols or for the sulfation of different nucleotides Km of nucleotides varied significantly. The concentration of PAP/PAPS in the cell is in μM range (Lin & Yang, 1998). However, in most cells, the total concentration of adenosine phosphates (AMP, ADP or ATP) is usually in the range 2 to 10 mM (p.287, Zubay, 1983). This indicates that PST may use AMP as a substrate or cofactor as effective as that of using PAP. Kds listed in Table 1 indicates that phenol sulfotransferase may be tightly bound to the many nucleotides in vivo. It is known that binding of PAP induce conformational change of phenol sulfotransferase (Yang et al., 1996) and ternary complex of E-PAP-phenols has been proposed as the inhibitory pathways for the physiological reaction of phenol sulfotransferase. The effect of nucleotides on the enzyme conformation and inhibition is now under investigation.

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附件

(Selected abstracts presented at scientific meeting)

5th International ISSX Meeting, Cairns, Australia, October 25-29, 1998

Interactions Among Nucleotide, phenol and Sulfotransferase: Implication on the Mechanism of Sulfuryl Group Transfer

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Sulfation in biological system plays important roles in regulating structure and function of macromolecules, availability of hormones and neurotransmitters, activation and deactivation of xenobiotics, and elimination of end products of catabolism. Sulfotransferases catalyze all the known biological sulfation which involves the transfer of sulfuryl group from a common sulfate donor, 3'-phospho adenosine 5'-phosphosulfate (PAPS). 3'-phospho adenosine 5'-phosphate (PAP) is the product of biological sulfation as well as cofactor of sulfuryl group transfer between sulfated and non-sulfated substrate of sulfotransferases. Addition of PAP into the mixture of phenol sulfotransferase and 4-nitrophenol (pNP) results in a new pNP spectrum (λmax = 375 nm). This finding indicates interaction among enzyme, PAP and pNP and provides us a tool for the study of their relationship. Circular dichroism and fluorescence spectroscopies are also used to investigate the spectral differences of enzyme, enzyme-nucleotide binary and enzyme-nucleotide-phenol ternary complexes. We propose that the absorption shift from 320 to 375 nm is mainly due to the presence of an ionized pNP in a mostly hydrophobic environment and that this charged compound is stabilized by a basic amino acid residue. Adenosine 5'-monophosphate, Adenosine 2',3'-cycliphosphate 5'-phosphate and Adenosine 2'-5'-bisphosphate were shown to replace PAP as cofactor for sulfuryl group transfer. Site-directed mutagenesis is used to study binding and activity of phenol sulfotransferase with PAP and other nucleotides. We proposed that positive charges at K65 and R68 of phenol sulfotransferase may be important for the binding of PAP by interacting with its 3'-phosphate. The mecanisms of sulfuryl group transfer based on the interactions among phenol, nucleotide and phenol sulfotransferase are proposed.

1998 Fall Camp, Enzymes: Function and Structure, The Society for Biochemistry and Molecular Biology, Chitou, Taiwan, October 30-November 1, 1998

SULFATION OF NUCLEOTIDES CATALYZED BY PHENOL SULFOTRANSFERASE.

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All sulfotransferases require nucleotides as cofactor or cosubstrate to catalyze sulfuryl group transfer. 3'-Phospho adenosine 5'-phosphate (PAP) or 3'-phospho 5'-phosphosulfate (PAPS) can serve as cofactor of phenol sulfotransferase (PST) for

transferring sulfuryl group from 4-nitrophenyl sulfate (pNPS) to a variety of phenols. We found that the release of sulfuryl group from pNPS can be catalyzed by PST in the presence of several nucleotides beside PAP and PAPS. The sulfated nucleotides were identified by ^{31}P NMR and HPLC. Kinetic data indicates that even adenosine 5'-monophosphate (AMP) and adenosine 5'-bisphosphate (ADP) can be used by PST to replace PAP. Km and Vmax of several nucleotides that used by PST as cofactor or cosubstrate were determined. In this presentation, we demonstrate that PST uses a broad range of nucleotides as substrate or cofactor for sulfuryl group transfer. The mechanism for sulfuryl group transfer is proposed.

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THERMOPHILICITY OF PIG LIVER IMIDASE

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Imidase is an enzyme with broad substrate specificity and also known as hydantoinase or dihydropyrimidinase. Hydrolysis of dihydropyrimidine is the only known physiological function of imidase. *In vitro* data indicates that many xenobiotics are better substrates than dihydropyrimidine. We found that imidase also prefer a much higher reaction temperature than that of its physiological condition. Imidase activities from different mammalian liver were measured at 25 and 50 °C and the enzyme activity from pig liver is the most temperature dependent. To study the thermoproperties of imidase, we purified pig liver imidase 2500 fold to homogeneity without heat treatment. It is essential to purify imidase without the generally used heat treatment for our interest is to identify the possible existence of both thermostable and thermolabile imidase. Only one imidase were purified with molecular mass of 307K detrmined by gel filtration and subunit mas of 51K determined by SDSPAGE in reducing condition. The enzyme is very stable at 60 °C and prefer to catalyzed imide hydrolysis above this temperature. The temperature effect on the Km and Vmax of imidase and the proposed mechanism of imide hydrolysis is presented.