行政院國家科學委員會補助專題研究計畫成果報告

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※ 由酚亞硫酸基轉移酵素之結構與功能 ※

※ 探討管制催化亞硫酸基轉移之機制 ※

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由酚亞硫酸基轉移酵素之結構與功能

探討管制催化亞硫酸基轉移之機制

The Structure/Function Relationship of Phenol Sulfotransferase
Regulation and Mechanism of Sulfuryl Group Transfer

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中文摘要

藉由數種實驗結果，包括：核酸與酚亞硫酸基轉移酵素(PST)**之親和性標記**(affinity labeling)**，氧化對於此酵素生理與轉移反應之不同影響**(-SH/-S-S-)**，酵素結構及其分子模擬**(Estrogen ST as template)**，定點突變所得之突變株酵素特性**(C66S, K65E&R68G)**，及** PST **之雙硫鍵位置；我們對於核酸**(3'-phospho adenosine 5'-phosphate, PAP)**與** PST **之反應機制與催化管制有進一步的了解。亞硫酸基轉移於生物系統中有極重要的功能，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。催化亞硫酸基轉移的關鍵在於酵素與核酸的相互作用，因為，所有的生物性亞硫酸基轉移反應都需有核酸硫酸化的過程(亦即產生** 3'-phospho adenosine 5'-phosulfate, PAPS) **。本計畫將運用上述各種方法，探討** PST **如何應用其結構上的變化來催化與調控亞硫酸基轉移反應，並進一步設計出使用不同核酸的新亞硫酸基轉移酵素。目前我們的研究發現亞硫酸基轉移酵素中有一形態不穩定區(主要包括** 63-Leu-Glu-Lys-Cys-Gly-Arg-68)**，雖然依氨基酸序列比對與結構分析結果，均與核酸之結合無直接相關，但此區的氨基酸卻可被核酸與** PST **之親和性標記。由分子模擬看出，此形態不穩定區位於核酸進出口，而且在此區的氨基酸改變後，嚴重影響到** PAP/PAPS **與** PST **的結合與釋放。**

關鍵詞：亞硫酸基轉移酵素；分子模擬；化學變性；定點突變；基因選殖與表達；酵素反應機制。

Abstract

Experimental results from other laboratories and ours have improved our understanding in the regulation and mechanism of sulfuryl group transfer catalyzed by sulfotransferase. These recent advances including affinity labeling of phenol sulfotransferase (PST), effect of oxidation/reduction on the PST activity, determination of the X-ray structures and molecular modeling of sulfotransferases, site-directed mutagenesis of sulfotransferases, and the determination of the position of disulfide bond on PST. Sulfuryl group transfer 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. The key step of sulfuryl group transfer in biological system is the interaction of 3'-phospho adenosine 5'-phosphate (PAP) with sulfotransferase which require 3'-phospho adenosine 5'-phosphosulfate (PAPS) as sulfuryl group donor in physiological condition. This proposal uses the established procedures

above to study the regulation and mechanism of PST catalyzed reactions. A flexible region (which includes 63-Leu-Glu-Lys-Cys-Gly-Arg-68) of PST is found to be important for the binding and release of nucleotides. Although sequence comparison and structural information indicate no direct involvement with PAP binding, mutations on this region find an opposite result. Affinity labeling with PAP analog also finds direct contact of this flexible region with nucleotide. This region is proposed to be the door of nucleotide binding site of PST and are found to be in the way of the exit of nucleotide by the analysis of molecular modeling.
Keywords: Sulfation; Nucleotide; Sulfotransferase; enzyme mechanism; 3'-Phospho Adenosine 5'-Phosphate (PAP); 3'-Phospho Adenosine 5'-Phosphosulfate (PAPS).

緣由與目的

Sulfuryl transfer reactions are widely observed in various biological processes [Jakoby & Ziegler, 1990]. Cytosolic sulfotransferases catalyze the transfer of the sulfuryl group from the ubiquitous cofactor 3'-phosphate 5'-phosphosulfate (PAPS[1]) to numerous substrates including steroids, bioamines (e.g. dopamine and catecholamine), therapeutic drugs, and environmental chemicals [Glatt, 2000]. In some cases, however, xenobitic sulfation can be used for the *in vivo* activation of prodrugs or can result in potentiating toxicity and carcinogenicity [Glatt, 1997; Meisheri *et al.*, 1998].

Phenol sulfotransferase [EC 2.8.2.9] [Mattock & Jones, 1970] contains five cysteine residues for each monomer of the homodimeric protein [Marshall *et al.*, 1998]. Previous study demonstrated that a highly conserved C66 is important for the regulation of PST activity through oxidation [Marshall *et al.*, 1997; Marshall *et al.*, 1998]. This cysteine has also been suggested to involve in the binding of the nucleotide substrate, since it was found that ATP-dialdehyde, a PAP analogue and affinity label, was covalently linked to the rat enzyme at C66 and the adjacent Lys 65 [Zheng *et al.*, 1994]. However, the crystal structure of sulfotransferase [Kakuta *et al.*, 1997; Kakuta *et al.*, 1998; Kakuta *et al.*, 1999; Bidwell *et al.*, 1999; Dajani *et al.*, 1999; Pedersen *et al.*, 2000] cannot confirm the interaction of C66 with PAP/PAPS binding site. It is interesting that the fragment of PST, from residues 63 to 68, cannot be resolved in several crystal structure of sulfotransferase [Kakuta *et al.*, 1997; Kakuta *et al.*, 1998; Kakuta *et al.*, 1999; Bidwell *et al.*, 1999; Dajani *et al.*, 1999; Pedersen *et al.*, 2000].

Although at least two cysteines are required to form an intermolecular disulfide bond, the effect of each cysteine on PST activity in different redox conditions has been demonstrated with a single cysteine mutant [Marshall *et al.*, 1997; Marshall *et al.*, 1998]. In this report, we constructed mutants that contain only two of the cysteines that are most likely to form intermolecular disulfide bonds. We are trying to understand the mechanism of the redox regulation and to explain why the increase of the physiological activity was observed at the expense of the transfer activity [Marshall *et al.*, 1997]. These mutants have provided us important information about the enzyme's activity brought about by redox system of the cysteine residues of phenol sulfotransferase and how the oxidation of cysteines results in the change of PST activity.

結果與討論

In this study, we have investigated the roles played by cysteine residues in redox environment, especially for that of C66 and C232, which have been proposed to form intra-molecular disulfide bond. We design a series of four PST mutants to verify this proposed model and to propose a mechanism that explains how the formation of disulfide bond may regulate the activity of PST. Three of the mutants, C232SC283SC289S, C82SC283SC289S and C66SC283SC289S, are designed to keep two of the three cysteines, C66, C82 and C232, that are found mutually nearby and the remaining

cysteines for these mutants are C66C82, C66C232, C82C232 and C66C283C289, respectively. The other mutant, C82SC232S which contains C66 but not C82 and C232, was used to test the function of C66 in the absence of other near by cysteines and is good comparison for a previously constructed mutant, C66S, to study the activity of PST in the absence of C82 and C232. These mutations allow us to elucidate the interaction among C66, C82 and C232 and the mechanism that result in the change of enzymatic activity.

The difference in kinetic mechanism of physiological and the transfer reaction involve the necessity of the release of PAP/PAPS during the course of sulfuryl group transfer [Yang *et al.*, 1996; Yang *et al.*, 1998). Thus, Kd of PAP and PST may have direct correlation with these PST catalyzed reactions. As shown in Table I, not all the Kds of PST and its mutant with PAP are affected in different redox environment. In a separate experiment, we found that PST was protected by PAP (data not shown) from oxidative reagent. As expected, Kd of α-form of PST, which contains a tightly bound PAP, is not affected in the different redox condition used in this study and served as a control for this experiment. Data of Table I indicates that changes of Kd for PAP and PST require the presence of both C66 and C232 in the enzyme. In such case, Kd1 of PAP and enzyme, for wild type PST-β and C82SC283SC289S only, in oxidative condition is ten thousand times higher than that in the reducing environment. This observation indicates that the change of PST activity upon oxidation may be due the change of nucleotide binding efficiency.

Kinetic data from the PST catalyzed transfer reaction also shows that redox effect and enzymatic activity are strongly dependent on the presence of cysteines in PST as shown in Tables II and III. Specific activities (Table II) for the transfer reaction were significantly reduced for only PST-β and C82SC283SC289S, the only two enzymes that contain both C66 and C232. The main reason for the decrease in specificities is due to the increase of Km and not the decrease of Vmax following oxidation as shown in Table III. The specific activity of the transfer reaction was not affected with a single mutation for each of the five cysteines except for C232 [Marshall *et al.*, 1997]. However, double or triple cysteine mutants resulted in the significantly decrease of the specific activities of the enzymes (Table II) and Vmax (Table III) of the transfer assay. Thus, C82, C283 and C289 are important for enzyme activity even though they may not directly involve in the redox regulation of PST. This explain the less significant redox effect on C82SC283SC289S compared to that of PST-β as shown in Table III. This observation is in consistent with the previous findings [Marshall *et al.*, 1997] that extending oxidation, which may modify all the cysteines, eliminates all the PST activity.

On the contrary to that of the transfer reaction, the oxidation of sulfotransferase may improve the Vmax of the physiological or reverse physiological reaction at the expense of increasing Km of PAP (Tables II and IV). The physiological or its reverse reaction of sulfotransferase requires the release of PAP (or PAPS) to continue for the next run of reaction [Yang *et al.*, 1996; Yang *et al.*, 1998]. Again, most significant change of Km of PAP was observed for PST-β and its mutant, C82SC283SC289S (Table IV).

The missing of the structure of C66 and its neighboring region in the crystal structure of sulfotransferase [Kakuta *et al.*, 1997; Kakuta *et al.*, 1998; Kakuta *et al.*, 1999; Bidwell *et al.*, 1999; Dajani *et al.*, 1999; Pedersen *et al.*, 2000] indicates that this region is likely to be a flexible loop. This flexible region must near the PAP binding site according to the labeling experiment [Zheng *et al.*, 1994] by a PAP analogue (ATP-dialdehyde). Together with the current and previous studies as discussed above, the mechanism of redox regulation of PST catalyzed reaction is proposed and illustrated in Schemes II and III. The release of PAP from PST is not required

throughout the transfer reaction as shown in Scheme II. In the reducing condition, nucleotide is retained in the enzyme for the flexible loop near PAP binding site is not removed as depicted in Scheme II(a). Upon oxidation, as shown in Scheme II(b), a disulfide bond between C66 and C232 is form and the flexible loop is removed to facilitate the in and out of the nucleotides. This cartoon explains why the nucleotide is tightly bound to PST in reducing condition and why incubating with GSSG results in the significant increase (10,000 folds) of Kd for only PST-β and C82SC283SC289S, which contain both C66 and C232.

Assuming that the opening of the flexible loop region upon oxidation affect only the binding of PAP, Scheme II would predict that Km of PAP may significantly increase while Vmax may remain relatively constant upon oxidation. However, multiple mutation of cysteine affects the activity of PST as shown in Tables II, III and IV. Thus, the change of Km and Vmax in different redox condition may be complicated and affected by the cysteines that do not directly involve in the formation of intra-molecular disulfide bond. Nevertheless, PST-β and C82SC283SC289S still show the most significant change of Km of PAP for the transfer reaction upon oxidation as shown in Tables II and III.

Similar mechanism is proposed for the physiological reaction catalyzed by PST as diagramed in Scheme III. The activity of oxidized form of the protein shows a 10-fold increase in physiological activity over the reduced form when assayed using 4-nitrophenol as acceptor [Marshall *et al.*, 1997]. It is also known that PAP is a product inhibitor for sulfotransferase catalyzed physiological reaction [Adams & Poulos, 1967; Adams *et al.*, 1974] and the release of PAP may be the rate-limiting step [Yang *et al.* 1998; Marshall *et al.*, 2000]. The above description in the reducing condition is illustrated in Scheme III(a) that release of nucleotides are hindered by a flexible loop region and is out of the way upon oxidation, as shown in Scheme III(b). The diagram explains why oxidation increase Km and eliminate the rate limiting step of the physiological reaction by significantly decrease the affinity of nucleotides and PST.

In conclusion, we propose that a flexible fragment of PST, from residues 63 to 68, affect PAP binding. The mechanism involves a flexible loop function as a door that may control the in and out of PAP. The door is closed in reducing environment and open in oxidative condition with the formation of an intra-molecular disulfide bond between C66 and C232.

Two possible pathways have been proposed to alter the PST activity in redox condition [Marshall *et al.,* 2000]. The other pathway involves an inter-molecular disulfide bond with C66. The mechanism of this pathway is being studied. Our data indicates that modification of C66 region reduce affinity of PAP and PST and gives similar changes of PST properties.

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