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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行政院國家科學委員會專題研究計畫成果報告

酚亞硫酸基轉移酵素催化反應之管制與機制
Regulation and Mechanism of Sulfuryl Group Transfer Catalyzed by Phenol Sulfotransferase

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中文摘要

生物體中藉由硫酸化來進行調控的物質有蛋白質、醣蛋白、醣類、荷爾蒙、神經傳導物質及包括藥物及致癌物等化合物；因此，亞硫酸基轉移參與生物訊息之分辨與傳遞、荷爾蒙之調節、解毒作用及調節神經傳導物質等重要生物反應。這些重要的生化反應都需由各種亞硫酸基轉移酵素催化，然而雖然有極重要的功能，我們對於此酵素反應機制的了解卻非常有限。藉由數種實驗結果，我們開始了解此酵素之反應機制與催化管制。相關實驗包括：核酸與酵素之親和性標記，氧化對於此酵素生理與轉移反應之不同影響，酵素結構及其分子模擬，定點突變所得之突變株酵素特性，及此酵素之雙硫鍵位置。本計畫將運用上述各種方法，探討此酵素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的結果，此區均與核酸之結合與催化反應無直接相關，但其氨基酸卻可被核酸與酵素之親和性標記。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，嚴重影響到核酸與酵素的結合與釋放。酚亞硫酸基轉移酵素內之雙硫鍵形成後，生理反應活性(Vmax)逐漸增加，亞硫酸基轉移反應(非生理反應)則僅 Km 增加。據此，我們設計出抗氧化之酚亞硫酸基轉移酵素，而且改善了其催化效率。酵素分子模擬顯示 C66,C82 及 C232 這三個胺基酸位置相當接近，可形成雙硫鍵；此外，K65 與 R68 經過定點突變後，也合乎根據酵素與反應物結合之三度空間立體結構所提出之反應機制。

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

Abstract

Many biologically important compounds such as hormones, polysaccharides, proteins, glycoproteins, neurotransmitters, many drugs and xenobiotics, are known to involve in sulfation and hydrolysis of their sulfate esters. Thus, 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. A large family of sulfotransferases are responsible for all the known biological sulfation. The enzymatic mechanism of sulfuryl group transfer has not been well understood. Results from the following experiments provide us clues to understand how the enzyme is regulated. They include affinity labeling of phenol sulfotransferase (PST), effect of oxidation/reduction on the PST activity, determination of the X-ray structures and molecular modeling of PST,

site-directed mutagenesis of PST, and the determination of the position of disulfide bond on PST. 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. Formation of disulfide bonds in PST increases its physiological activity. However, initial oxidation of PST does not affect its transfer activity. Both physiological and transfer activity disappear upon further oxidation of PST. We have put together the information from mechanism of PST action, molecular modeling, chemical modification and site-directed mutagenesis of PST to design several novel enzymes with improved properties.

Keywords: Sulfotransferase; sulfation; molecular modeling; chemical modification; anti-oxidation; site-directed mutagenesis, gene cloning and expression, enzyme mechanism.

緣由與目的

Sulfuryl transfer reaction is widely observed in various biological processes (*1*). 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 (*2*). In some cases xenobitic sulfation can be used for the *in vivo* activation of prodrugs or can result in potentiating toxicity and carcinogenicity (*3, 4*).

Rat phenol sulfotransferase [EC 2.8.2.9] (*5*) contains five cysteine residues in each monomer of the homodimeric protein (*6*). Previous study demonstrated that a highly conserved C66 is important for the regulation of PST activity through oxidation (*7, 8*). 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 Cys 66 and the adjacent Lys 65 (*9*). However, the crystal structure of sulfotransferase (*10-15*) cannot confirm the interaction of Cys 66 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 (*10-15*).

The effect of each cysteine on PST activity in different redox conditions has been demonstrated with a single cysteine mutant (*7,8*). In this report, we constructed mutants that contain only two of the cysteines that are likely to form intramolecular 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 (*7*). 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 allow us to elucidate the interaction among C66, C82 and C232 and the mechanism that result in the change of enzymatic activity.

結果與討論

Construction, Expression and purification of recombinant PST –To study the interaction of cysteines under different redox conditions, PST mutants, C232S-C283S-C289S, C82S-C283S-C289S, C66S-C283S-C289S and C82S-C232S, were prepared by site-directed mutagenesis. Together with C66S constructued previously (7), the remaining cysteines in PST for these mutants are C66-C82, C66-C232, C82-C232

C66-C283-C289, and C82-C232-C283-C289, respectively. Thus, the structural and functional relationship of cysteine residues 66, 82 and 232 could be evaluated.

Recombinant wild type and mutant PSTs were overexpressed and isolated from *E. coli* strain BL21 (DE3) bearing the pET3c or pET23a(+) vector with proper insert as shown in Table 1. The PST expression is signifcantly influenced by the mutation, especially for C66S-C283S-C289S and C232S-C283S-C289S that were not highly expressed in *E. coli*. All enzymes studied were purified and the purity of PST and its mutants was shown by SDS-PAGE at 34-kDa to at least 95% homogeneity.

Effect of redox on dissociation constants – Dissociation constants of PAP and PST (β form) increase four orders of magnitude in the presence of GSSG. As shown in Table 2, only mutant C82S-C283S-C289S, the only mutant contained both C66 and C232, exhibits oxidation effect on the dissociation constants with PAP. Both K_d1 and K_d2 of all other cysteine mutants remained unchanged to redox using TCEP and GSG for reduction and oxidation, respectively. Mutant C66S-C283S-C289S that was not active under standard assay condition was found to be associated with PAP tightly.

Effect of redox on the activity of recombinant PSTs – Specific activities of PST and its cysteine mutants were listed in Table 3. Significant change of specific activity in different redox status was observed for wild type (β form) and C82S-C283S-C289S mutant. Both enzymes retained cysteine residues at 66 and 232. Mutation of more than one cysteine significantly lowered the PST activity.

Effect of redox on V_{max}, K_m and ratio of catalytic efficiency – The dependence of V_{max}, K_m and ratio of catalytic efficiency under different redox conditions were varied among wild type and mutant PSTs as shown in Tables 4 and 5. The most significant variation of enzyme activity for the transfer reaction under different redox condition was K_m (one thosand fold difference) for β-form of wild type PST (Table 4). Without affecting much of the enzyme activity, mutation at C66 (C66S) eliminates most of this redox effect. In general, K_m of PAP for the transfer reaction was increased under oxidation condition with the exception of the mutant C82S-C232S (Table 4), but significant increase of K_m was observed for C82S-C283S-C289S. The ratio of catalytic efficiency under reductive and oxidative condition showed that replacement of cysteine significantly reduced the sensitivity for redox effect. The results indicated that, in addition to function as redox regulators, cysteines were important for maintaining the PST activity.

When PAP was used as a substrate for the reverse physiological reaction, a different redox effect on the PST activity was observed as shown in Table 5. However, it is still the wild type (β form) and C82S-C283S-C289S that were most sensitive to the redox effect. It is particularly notable that K_m of PAP for C82S-C283S-C289S increased 40 times after oxidation (Table 5) greater than that of wild type. Unlike that of the transfer reaction, the V_{max} of wild type PST increased following incubation with GSSG.

Reactivation of the transfer activity with TCEP- The redox regulation of PST activity was reversible as shown in Figure 1. PST and its cysteine mutants were found to be stable in reducing condition with the exception of C82S-C283S-C289S. Mutation at cysteine 66 (C66S) resulted in the stabilization of PST under oxidation condition. Mutation of all other cysteines, C82, C232, C 283 and C 289, did not help to stabilize PST in the presence of GSSG. Mutation at cysteines at 283 and 289 instabilized PST even under reducing condition.

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