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(I) BINDING ENERGY AND THE MECHANISM OF SULFURYL GROUP TRANSFER

(II) MECHANISM OF IMIDASE - A THERMOPHILIC ENZYME FROM PIG LIVER

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Abstract:

(I) The first step of enzymatic reaction is generally the binding of substrates so that enzyme can provide a special environment for reactants and facilitate the reaction through pathways that are not allowed in a uncatalyzed condition. Phenol sulfotransferase exhibits unique properties for the binding of PAP and phenols that can be observed by a variety of spectroscopic methods for the study of the mechanism of the sulfuryl transfer. Specifically, this report will include: binding, reaction and inhibition of PAP and its analogues; function of each cosubstrate binding sites; the use of mutant of phenol sulfotransferase to study the function of cosubstrate binding sites; and the characterization phenol binding site. We are trying to use the data collected from this proposal to understand and clarify the following: 1. Why two cosubstrates binding sites are necessary for sulfuryl group transfer? 2. How the catalytic efficiency of the sulfotransferase affected by the tight binding of PAP? 3. Are the two cosubstrates binding sites used for different reactions that catalyzed by sulfotransferase? 4. Reexamine the inconsistency of the kinetic mechanisms that were proposed and compare with recent finding about cosubstrate binding. Our long term goal is to fully understand chemical mechanism of sulfotransferase and to construct a free energy profile for the sulfuryl group transfer reaction. With this information, we would like to design mutant enzyme with artificial properties.

(II) We purified an imidase to homogeneity from pig liver and determined that it is thermophilic. This enzyme prefers to catalyze hydrolysis of imides above 60 蓍 rather than at around the body temperature of pig. Mammalian imidase catalyzes the hydrolysis and synthesis of a variety of imides, dihydropyrimidines and hydantoins. In addition to its broad substrate spectrum, imidase uses different reaction conditions for different substrates. We explore how imidase adapts to the variation of conditions, such as temperature, pH and substrate specificities for reaction. We have proposed mechanism for the hydrolysis and synthesis of imides base on the structure/function relationships of the imide substrates. Further investigation of the mechanism needs to be clarified to understand how enzyme catalyzes synthesis and hydrolysis of imides. Another approach to understand the action of imidase is from the direction of enzyme evolution. To understand weather thermophilicity of imidase inherit from the ancient enzymes that are believed to have to work in an extreme

environment; we also compare amino acid and DNA sequences of imidases and related enzymes from different sources and analyze their relationship with thermophilicity and reaction mechanism.

Keywords:
(I) Binding energy; 3'-Phospho Adenosine 5'-Phosphate (PAP); 3'-Phospho Adenosine 5'-Phosphosulfate (PAPS); p-Nitrophenyl sulfate (pNPS); Sulfuryl Group Transfer; Sulfotransferase.
(II)thermophilic enzyme; imidase; enzyme mechanism

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(I) Tyrosine-ester sulfotransferase catalyzes reversible sulfuryl group transfer among 3'-phospho adenosine 5'-phosphate (PAP) and a variety of phenols, amines and hydroxyl amines. Chemical mechanism of sulfuryl group transfer by tyrosine-ester sulfotransferase (phenol sulfotransferase IV) was found to be similar to acid catalyzed non-enzymatice reaction (Duffel and Jakoby, 1981). This mechanism will require a hydrophobic active site which has been proposed recently (Yang et al., 1997). Even though it is well known that 4-nitrophenol (pNP) or other phenols are inhibitors of phenol sulfotransferase (2), the mechanism involved remains unclear. We have previously observed a unique absorption at 375 nm from E-PAP-pNP ternary complex (Yang et al., 1997). Based on the relevant spectroscopic data both in solution and sulfotransferase, we propose that the absorption shift is mainly due to the presence of an ionized pNP which is stabilized by a basic residue (probably a lysine) in a mostly hydrophobic environment.

(II)*Differences in the imidase activity from liver extracts of mammals*

Imidase activity at temperature much higher than the body temperature of the host was still found as shown in Table 1. The liver extracts were heated at 60 蓄 for 20 min and centrifuged before assaying for imidase. This procedure minimizes the precipitation of protein at elevated temperature that may interfere the observation of absorption change at 298 nm.

Imidase activity at either 25 or 50 ℃ shows little differnet before and after heat treatment. It inidicates that most of the imidase activity measured comes from heat stable enzymes. Imidase activitives from all the animals tested in standard condition increase about two to six fold when temperature increase from 25 to 50 ℃. Preliminary data from Table I indicates that imidases from different animal sources may differ significantly in both quantity and property. This result leads us to the decision that theromophilic imidase may be best obtained from pig liver whose activity shows the most temperature dependent.

Various sources of imidase activity from pig liver were also tested. Gender does not show significant difference in imidase activity. Howerer, young pig liver (2 day old) contains only about 10% imidase activity compared to that of two weeks old or older. The storage of pig liver also affect little on the imidase activity. Imidase activities from fresh (several hours at room temperature or storage at 4 - 8 ℃ but not frozen) or frozen pig liver before and after heat treatment show little different.

Purification of imidase from pig liver

Imidase from pig liver was purified about 2,500-fold with 15% yield to produce a homogeneous protein by the criteria of SDS-gel electrophoresis (Figure 1 and Table II). A heat-treatment procedure was deliberately avoided during the purificaiton so that we would not inactivate possible existence of thermolabile imidase. The heat-treatment was found very useful and used to purify other similar enzyme from rat (10), calf (8) and pig (9) livers. Omission of this procedure for the purification of imidase form rat liver required a tediously large number of procedures to remove the minor contaminants that were visible on SDS-gels (1). We are able to develop a different purification scheme without heat-treatment and still obtain resonable yield. Since we are interested in the temperature effect on imidase, it is important to prepare a homogeneous enzmye without previous experience of heat much higher than its original environment. Also, a different thermolabile imidase, if exist, should be observed with this purification scheme.

Homogeneity and size of the thermophilic imidase

The purity of purified imidase was shown by SDS-PAGE in Figure 1e. Molecular weight for a single polypeptide chain was estimated to be about 51,000. The molecular weight of imidase estimated by gel filtration is about 300K using thyroglobin (669K), ferritin (440K), catalase (232K) and aldolase (158K) as molecular weight standards.

Thermophilicity and thermostability of pig liver imidase

Purified imidase from pig liver exihibit thermophilicity as shown in Figure 2. Imidase activity continues to increase with the elavation of temperature up to 65 ℃ except for reaction at pH 6. The thermophilicity of imidase is limited by its thermostability and pH of the reaction condition at elevated temperature as shown in Figures 3 and 4. The stability of imidase decrease dramatically in acidic or akaline pH at 60 ℃ (Figure 3). At pH around 7.5 to 8.5, imidase is relatively stable at 50 ℃ or lower for over two hrs. The half life of imidase at 60 ℃ is only about 35 min (Figure 5) and decrease sharply at 65 ℃ for a half life of only 10 min. No enzyme activity can be observed when imidase was heated at 70 ℃ for less than 10 min. This result indicates that thermophilicity of imidase is mainly limited by its thermostability when pH is not a limiting factor.

Effect of pH on the thermostability and thermophilicity of imidase

Activity and stability of imidase is pH dependent and the this pH effect is temperature dependent as shown in Figures 3 and 4. Figure 3 shows that at acidic pH, imidase is stable at 40 ℃ but less stable at elevated temperature (50 and 60 ℃). This observation explain the less thermophilicity of imidase at acidic pH as shown in Figure 2. At lower temperature (below 40 ℃), imidase is stable in a wide range of pHs (Fig. 3), so its activity profile at 25 ℃ as shown in Figure 4 is not influenced by the stability of protein. However, pH profile at 60 ℃ (Fig. 4) is the mix result of imidase activity and stability due to the instability of imidase at acidic and alkaline pHs (Figure 3). The pH profiles of imidase at 25 and 60 ℃ deviate about 0.5 to 1 pH unit and pH optimun at 60 ℃ shifts to a more alkaline condition as shown in Figure 4. This shift of pH optimum is not due to the stability of imidase at extreme pHs for imidase at 60 ℃ is even less stable in alkaline conditionand than that in acidic conditon (Fig. 3). Thus, increase reaction temperature not only improve specificity activity of imidase but also change its pH optimum.

Effect of temperature on the substrate specificity of imidase

Three groups of substrates were tested to study the temperatrue effect on the substrate specificity of imidase as shown in Table III. We found significant variation on temperature dependent imidase activity when different substrates were used. Specific activity of imidase toward the only two know natural substrates of imidase showns the most temperature dependent. Hydrolysis of dihydrouracil and dihydrothymine increase 50 and 60 folds, respectively, when reaction temperature increase from 25 to 60 ℃. On the contrary, imidase catalyzed hydrolysis of hydantoins which is a five-member ring analog of dihydrouracil exihibits rather temperature insensitive. Only 2 and 3 folds increase of the imidase specificity for hydantoin and 1-methyl hydantoin, respectively, when reaction temperature increase from 25 to 60 ℃. Thus, while a poor substrate for imidase at 25 ℃, dihydrouracils become much better substrates at 60 ℃ than that of hydantoins (Table III). Effect of temperature on imides, phthalimide and glutarimide as substrates of imidase is also significant. Phthalimide is used as standard substrate and its temperature dependency was studied extensively as shown in Figures 2, 3 and 4 and Tables III and IV. Temperature effect of glutarimide as substrate of imidase is similar to that of phathalimide as shown in Tabes III and IV. The effect of temperature on the substrate specificity of imidase seems dependent on the functional group and structure of substrates.

Effect of temperature on the Km, Vmax and catalytic efficiency of imidase

Change of reaction temperature may chagne Km and Vmax of imidase and in turn affect the specifici activity shown in Talbe III. Table IV shows that temperature effect on these constants is substrate dependent. Neither Km nor Vmax of imidase is significantly affected when hydantoin is used as substrate at 25 or 60 ℃. This is a sharp contrast to that of imidase useing dihydrothymine or phthalilmide as substrate. Vmax of imidase increase one hundred fold when reaction temperature increase from 25 to 60 ℃ while Km also increase 20 to 30 folds. Overall catalytic efficiency of imidase also increase when phthalimde or dihydrothymine is used as substrate, but only around two to four folds. Due to the low solubility of imides and high Km value of imidase when hydantoin and phthalimide were used as substrate, the Km and Vmax was obtained with a large standard error. This is because the extrapolation from a small value of substrate concentration (in a tenth of mM range) to a large

number of Km (around 10 mM). However, it is very clear that at these available substrate concentrations, imidase activity increase in a almost linear fashion with the increase of the concentration of hydantoin at 25 and 60 蔣 and phthalimide at 60 蔣. Thus, it is certain that much higher value of Km and Vmax of imidase would be expected when its substrates are hydantoin at 25 and 60 蔣 and phthalimide at 60 蔣. Instability of imidase at elavated temperature may also contribute to the large standard error of these constantants measured at 60 蔣 when both phthalimide and hydantoin were used as substrates. Phthalimide is also unstable at elevated temperature. Spotaneous hydrolysis of 1 mM phthalimide at pH 7 is about 0.4 nmole/min at 25 蔣 and increase to 7 and 15 nmole/min when temperature is increse to 50 and 60 蔣, respectively. All other substrates, including glutaramide, mentioned in this manuscript are stable at their reaction conditon.

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I. Major research accomplishment:

1. Identificaiton and purificaiton to homogeneity of a thermophilic imidase from pig liver.

2. Study phenol binding environment of phenol sulfotransferase by new spectrum of 4-nitrophenol.

II. Other results

1.Two manuscripts in preparation.

2. Three M.S. thesis completed.

3. Seven reports in scientific meeting.

III. Self evaluation

Entering the forth year in the research of sulfotransferase and second year in that of imidase in my laboratory, we have established all the necessary tools for the study of these two enzyme. I expect fruitful results to come based on what we have already established. At present, I feel that we have accomplished our proposed research targets.

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(Selected abstracts presented at scientific meeting)

17th International Congress of Biochemistry and Molecular Biology, **San Francisco, California, August 24-29, 1997**

Reactions of Phenol Sulfotransferase with Nucleotides

Yang, Y.-S. and Lin, E.-S., *Institute of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan*

All sulfotransferases require adenosine 3',5'-bisphosphate (PAP) or 3'-phospho adenosine 5'-phosphosulfate (PAPS) to catalyze sulfuryl group transfer. Thus, PAP is used as cofactor by phenol sulfotransferase (PST) for moving sulfuryl group from p-nitrophenyl sulfate (pNPS) to other phenols. PST also tightly binds to a variety of nucleotides and we found that sulfuryl group can be released from pNPS in the presence of several nucleotides other than PAP or PAPS. Binding of nucleotides by PST is rather non-selective. We determined binding constants of a variety of nucleotides and examined their potential as cofactors or substrates of phenol sulfotransferase. Structure and functional groups of nucleotides and their requirements for PST binding are examined. We found that even ribose and adenine, two major components of the adenosine nucleotides, tightly bound to PST. Some nucleotides, such as adenosine 2', 5'-bisphosphate and AMP, accept and transfer sulfuryl group analogue to PAP with less efficiency. Other nucleotides, such as ADP, involve in both sulfuryl group transfer and hydrolysis of phospho ester after sulfation. While unable to hydrolyze pNPS directly, PST was found to hydrolyze PAPS at alkaline pH. Kinetic data and NMR are used to determine the reaction pathways of PST catalyzed sulfation and hydrolysis. In this presentation, we would like to demonstrate that PST uses a broad range of nucleotides as substrate or cofactor for sulfuryl group transfer. (Supported by Grant NSC 86-2311-B-009-002 of National Science Council, Taiwan)

_ _璨_|86 _| , December 5-7, 1997, Hsinchu, Taiwan

How Phenol Sulfotransferase Alters Its Substrate Specificities?
[Invited Lecture]

Yang, Y.-S., *Institute of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan*

Phenol sulfotransferase catalyze sulfuryl group transfer from 3'-phospho adenosine 5'-phosphosulfate (PAPS) to a variety of compounds with distinct structures and functional groups. The selection of the sulfuryl group acceptor was found to be dependent on the reaction condition. We propose that different conformation of the enzyme may be responsible for the wide spectrum of substrate specificities. Over-expression of a single cDNA of rat liver phenol sulfotransferase produces two enzyme forms with different catalytic activities and is dependent on the availability of 3'-phospho adenosine 5'-phosphate (PAP). Evidences are presented to show that PAP and other nucleotide cofactors affect the

conformation and phenol binding environment of phenol sulfotransferase. Mechanisms and pathways of inhibition and substrate specificity of the enzyme action are proposed.

The Thirteenth Joint Annual Confernece of Biomedical Sciences, Taipei, Taiwan, April 18-19, 1998

Binding and Reaction of Nucleotides with Phenol Sulfotransferase

Lin, E.-S., Su, T.-M. and Yang, Y.-S., *Institute of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan*

Sulfuryl group transfer catalyzed by sulfotransferases require adenosine 3',5'-bisphosphate (PAP) or 3'-phospho adenosine 5'-phosphosulfate (PAPS) as cofactor or cosubstrate, respectively. Phenol sulfotransferase (PST) tightly binds to a variety of other nucleotides, and we found that sulfuryl group can be released from 4-nitrophenyl sulfate in the presence of several nucleotides other than PAP or PAPS. The binding of nucleotides by PST is rather non-selective. We have determined binding constants of a variety of nucleotides and examined their potential as cofactors or substrates of phenol sulfotransferase. Structure and functional groups of nucleotides and their requirements for PST binding have been examined. On the contrary, reaction of nucleotides with PST is very selective. We propose that PST conformational change induced by nucleotide binding may be responsible for the activity of the enzyme. Fluorescence and CD spectra of PST and PST-nucleotide binary complex will be presented to support our proposal.

The Fourth Symposium on Recent Advances in Biophysics, Taipei, Taiwan, May 13-16, 1998

Engineering Nucleotide Binding Site of Phenol Sulfotransferase

Tsan, S.G. and Yang, Y.-S., *Institute of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan*

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, a nucleotide called 3'-phospho adenosine 5'-phosphosulfate (PAPS). Another nucleotide, 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. Site-directed mutagenesis was used to study binding and activity of phenol sulfotransferase (PST) with PAP and other nucleotides. Amino acids (Lys65 and Arg68) of PST binding region, previously identified by PAPS analogs, were mutated and mutant enzymes of different specificities toward a variety of nucleotides were obtained. A double mutant (K65E+R68G) exhibits over 500 fold increase in Km for PAP with Vmax remained the same. Adenosine 5'-monophosphate (AMP), Adenosine 2',3'-cycliphosphate 5'-phosphate and Adenosine 2'-5'-bisphosphate were shown to replace PAP as cofactor for sulfuryl group transfer. However, neither mutant Km nor Vmax of these

nucleotides differ significantly with that of wild type . AMP, without a 3'-phosphate as compared to PAP, is a cofactor of both mutant and wild type phenol sulfotransferase with exactly the same V/K value. 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. Another mutant (K65G) showed similar results.

The Fourth Symposium on Recent Advances in Biophysics, Taipei, Taiwan, May 13-16, 1998

Use 4-Nitrophenol as Probe for the Study of Phenol Binding Environment of Sulfotransfrase

Huang, C.-J. and Yang,Y.-S., *Institute of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan*

Addition of 3'-phospho adenosine 5'-phosphate (PAP) into the mixture of phenol sulfotransferase and 4-nitrophenol (pNP) results in the pNP spectral changes at 320 (decrease in absorption) and 375 (increase in absorption) nm at pH 6. This finding indicates interaction among enzyme, PAP and pNP. A ternary complex is form after pNP binds to an E-PAP binary complex. The new spectrum of pNP is not identical to the spectra of its protonated or deprototnated forms in aqueous solution. Thus, the spectral changes provide us an opportunity to study the binding environment of phenol binding site of phenol sulfotransferase. Spectrum of pNP may be affected by the polarity of solvents, the protonated state and the availability of hydrogen binding. We examined these effects in a model study using organic solvents to mimic the phenol binding environment of phenol sulfotransferase. A variety of different pNP spectra were obtained. Base on this information, we propose that the phenol binding site of sulfotransferase should be hydrophobic consistent with the fact that the function of this enzyme is to transform hydrophobic compounds to more hydrophilic ones. 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. We also determined binding stoichiometry of the enzyme, PAP and pNP and propose mechanism for the reaction and inhibition of phenol sulfotransferase with nucleotides and phenols.