Photoaffinity Labeling of Human Recombinant Sulfotransferases with 2-Azidoadenosine 3',5'-[5'-32P]Bisphosphate*

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Photoaffinity labeling with 2-azidoadenosine 3',5'-[5'-32P]bisphosphate was used to identify and characterize adenosine 3',5'-bisphosphate-binding proteins in human liver cytosol and recombinant sulfotransferase proteins. The sulfotransferases investigated in these studies were the human phenol sulfotransferases, HAST1, -3, and -4, dehydroepiandrosterone sulfotransferase, and estrogen sulfotransferase. The cDNAs for these enzymes have been previously cloned and expressed in COS-7 cells or Escherichia coli. Photoaffinity labeling of all proteins was highly dependent on UV irradiation, was protected by co-incubation with unlabeled adenosine 3',5'-bisphosphate and phosphoadenosine phosphosulfate, and reached saturation at concentrations above 10 μM. To verify that the 31–35-kDa photolabeled proteins were indeed sulfotransferases, specific antibodies known to recognize human sulfotransferases were used for Western blot analyses of photolabeled proteins. It was shown unequivocally that the proteins in the 31–35-kDa region recognized by the antibodies also photoincorporated 2-azidoadenosine 3',5'-[5'-32P]bisphosphate. This is the first application of photoaffinity labeling with 2-azidoadenosine 3',5'-[5'-32P]bisphosphate for the characterization of recombinant human sulfotransferases. Photoaffinity labeling will be also useful in the purification and functional identification of other adenosine 3',5'-bisphosphate-binding proteins and to determine amino acid sequences at or near their active sites.

Sulfation is an important pathway in the biotransformation of many drugs, xenobiotics, neurotransmitters, bile acids, and hormones. The sulfate donor for these reactions is 3'-phosphoadenosine 5'-phosphosulfate (PAPS). Adenosine 3',5'-bisphosphate (PAP) is a product of the reaction catalyzed by all sulfotransferases (STs) and competitively inhibits PAPS binding (1, 2).

Direct photoaffinity labeling with radioactively labeled PAPS has been used to identify PAPS-binding proteins. Lee et al. (3) used direct labeling with 3'-phosphoadenosine 5'-32P phosphosulfate to identify an M1, = 34,000 protein involved in PAPS translocation in Golgi membrane preparations of bovine adrenal medulla (3). Otterness et al. (4) utilized a similar approach, using [35S]PAPS for direct photoaffinity labeling of human liver STs. It was shown that UV irradiation of [35S]PAPS in the presence of partially purified human liver thermostable phenol sulfotransferase (PST) resulted in the labeling of 35-kDa proteins with properties identical to those of thermostable PST. These results indicated that [32P]PAP and [35S]PAPS can be used as direct photoaffinity ligands for the study of ST and other PAPS-dependent proteins; however, the reaction with the enzyme depends on high energy UV light activation of pyrimidine residues, and it is not always possible to distinguish covalent binding from nonspecific enzyme inactivation.

Another strategy for affinity labeling and probing nucleotide binding sites in protein molecules is to use photo reactive substrate analogs or inhibitors containing an azido group, such as 2-azidoadenosine and 8-azidoadenosine (5, 6). A new photo-reactive pyrimidine analog, 2-azidoadenosine 3',5'-[5'-32P]bisphosphate (2-azido-[32P]PAP), was synthesized by Sylvers et al. (7) and applied to the photoaffinity labeling of Escherichia coli ribosomes.

In this paper, we report photoaffinity labeling studies of human and rat liver cytosolic ST and several recombinant human STs with 2-azido-[32P]PAP synthesized and characterized according to Sylvers et al. (7). Studies were performed that demonstrated specific photoincision of 2-azido-[32P]PAP into these enzymes and also indicated the potential usefulness of this approach in the purification and molecular characterization of purified native and denatured PAPs- and PAP-binding proteins, including the STs. Moreover, photolabeling with 2-azido-[32P]PAP will be also useful in studies investigating the structure of the ST active sites.

MATERIALS AND METHODS

PAP, PAPS, 4-nitrophenol, and other reagents were purchased from Sigma. 2,6-Dichloro-4-nitrophenol (DCNP) was obtained from K and K Laboratories (Plainview, NY).

Synthesis of 2-Azidoadenosine 3',5'-[5'-32P]bisphosphate—2-Azido-[32P]PAP was synthesized and purified as described previously (7). [32P]ATP was from ICN (Irvine, Ca). The incorporation of 32P from ATP was usually over 70%, and the specific activity of 2-azido-[32P]PAP was 1–3 mCi/μmol.

Human and Rat Liver Cytosolic Preparation—Cytosolic fractions from male rat (Sprague-Dawley, 220–250 g) and human livers were prepared as described previously (8) and were rapidly frozen in liquid nitrogen and stored at −80 °C. The protein concentration was determined by the Bradford method (9), using bovine serum albumin as a standard.

cDNA Cloning and Expression in COS-7 Cells—Expression vectors
containing cDNAs encoding HAST1, HAST3, and HAST4 were transfected into COS-7 cells as described previously (10–12). The vectors contain SV40 sequences, which permit high level expression of individual STs. After 40–48 h, an aliquot of the transfected COS-7 cells was incubated in medium containing [35S]methionine before determining the amount of nascent enzyme synthesized by immunoadsorption and SDS-PAGE (13). The remaining transfected cells were stored at −70 °C in 50 mM Tris-HCl, pH 7.0, until assay.

To clarify nomenclature, HAST1 corresponds to p-PST or thermostable PST, HAST3 is m-PST or thermolabile PST, and HAST4 is a new form of PST differing from HAST1 by 12 amino acids. HAST4, unlike HAST1 and HAST3, is incapable of sulfating dopamine, and its Km, for 4-nitrophenol sulfation (74 μM)2 is markedly different from those of HAST1 (0.6 μM) and HAST3 (2200 μM) (12).

Bacterial Expression of Human DHEA-ST and EST—The human STs, DHEA-ST and EST, were expressed in E. coli as described previously (14) and partially purified using DEAE-Sepharose 4B chromatography (15).

Antibody Preparation—The characterization of specific rabbit anti-human DHEA-ST, EST, and PST polyclonal antibodies has been described previously (16–18), and these antibodies were used for immunoblot analysis of DHEA-ST and EST expressed in bacteria.

Photoaffinity Labeling—Human or rat liver cytosol (50 μg) or recombinant STs (10 μg) were suspended in 100 mM HEPES, pH 7.0, and 5 mM MgCl2 at 37 °C in a final volume of 25 μL. 2-Azido-[32P]PAP (200 μM, 2–5 mCi/μmol) was added to a final concentration of 10–40 μM, and allowed to equilibrate for 20 s, followed by UV irradiation with a hand-held UV lamp (UVP-11, 254 nm, Ultraviolet Products, Inc.) for 90 s at room temperature. For competition experiments, the appropriate unlabeled competing nucleotide was added to the reaction mixture just before addition of the probe. Any deviations from the above procedures are described in the appropriate figure legends. Reactions were terminated and processed for SDS-PAGE as described previously (19). Proteins were separated as described previously (19) using 12.5% gels, followed by autoradiography for 1 h to 2 days. In some cases, the separated proteins were transferred from the gel to nitrocellulose by electroblotting, and Western blot analysis was performed by the method of Towbin et al. (20). Autoradiographs were quantified using a Bio-Rad imaging densitometer.

RESULTS

Photoaffinity Labeling of PAP-binding Proteins in Cytosolic and Membrane Fractions—Cytosol and microsomal fractions from human and rat livers were irradiated in the presence of 40 μM 2-azido-[32P]PAP. Fig. 1 shows an autoradiograph of the PAP-binding proteins separated on SDS-PAGE. The labeling was strictly dependent on UV irradiation as evidenced by the absence of labeled proteins in non-irradiated samples (Fig. 1, lanes 1, 5, 8, and 12). With human liver microsomes, there was no significant specific photolabeling of proteins in the 30–40-kDa range (Fig. 1, lanes 2 and 3), and detergent treatment of the membranes resulted in no apparent increase in photolabeling (Fig. 1, lane 4). The labeling pattern in human cytosol, however, showed a high degree of specificity. Photolabeling of the human cytosolic fraction resulted in the specific labeling of two protein bands at approximately 32 and 34 kDa (Fig. 1, lane 6) corresponding to the established molecular masses of human cytosolic STs. Photoincorporation could be competitively inhibited by 200 μM concentrations of unlabeled PAP (Fig. 1, lane 7), and unlabeled PAPS inhibited photolabeling as well as PAP (data not shown). Protein bands at 31 and 40 kDa were also photolabeled, but the photoincorporation was not totally specific as evidenced by incomplete protection (31 kDa) or a lack of protection (40 kDa) in the presence of unlabeled PAP (Fig. 1, lane 7).

Several protein bands in rat liver microsomes photoincorporated 2-azido-[32P]PAP (Fig. 1, lane 9), but the photolabeling was inhibited by unlabeled PAP only in the approximately 40-kDa band, and even here protection was not complete (Fig. 1, lane 10). The only significant effect of detergent treatment of microsomes was to completely abolish photolabeling of a protein band of approximately 28 kDa, outside the mass range of the STs (Fig. 1, lane 11).

With cytosolic preparations from rat liver, on the other hand, proteins having molecular masses of 30–38 kDa, corresponding to the reported molecular masses of rat liver STs, were specifically photolabeled (Fig. 1, lane 13). Except for a band at approximately 40 kDa, the labeling was effectively inhibited by preincubation with 200 μM cold PAP (Fig. 1, lane 14). The pattern of rat liver cytosolic STs is different from the human ST pattern and is consistent with the presence of different forms of ST.

Concentration Dependence—The effect of increasing concentrations of 2-azido-[32P]PAP on photoincorporation into ST was investigated using HAST3 expressed in COS-7 cells. Concentrations from 0.1 to 50 μM were investigated. The extent of photoincorporation of the probe into the 34-kDa fraction in the HAST3 preparation increased with increasing concentrations of probe. As shown in Fig. 2, photolabeling of HAST3 by 2-azido-[32P]PAP was saturable, with half-maximal photoincorporation between 2.5 and 10 μM, as determined by densitometry. A concentration of 10 μM was chosen to optimize the quantity of radioactivity incorporated into the protein while at the same time minimizing the nonspecific background.

Inhibition by PAP and PAPS—To characterize the specificity of the photolabeling reaction, the effect of unlabeled PAP and PAPS was examined by adding the corresponding nucleotide over a range of concentrations from 10 to 50 μM. Photoincorporation of 2-azido-[32P]PAP into HAST3 was found to be inhibited in a dose-dependent manner by both nucleotides (Fig. 3), with PAP (lanes 2–4) being somewhat more effective than PAPS (lanes 5–7).

Effect of 4-Nitrophenol and DCNP—COS-7 expressed HAST3 is enzymatically active in sulfating 4-nitrophenol, a model substrate for the phenol-sulfating form of human sulfotransferases, and is inhibited by the ST inhibitor, DCNP.

2 M. E. McManus, unpublished data.
Therefore, the effect of both compounds on photolabeling of HAST3 by 2-azido-[32P]PAP was investigated. As shown in Fig. 3 (lanes 8–10), 4-nitrophenol was not required for photolabeling of the 34-kDa protein with 2-azido-[32P]PAP. In fact, 100 μM concentrations of 4-nitrophenol inhibited photolabeling of the 34-kDa protein (lanes 3, 11–13). Although it may not be evident from the figure, because of the exposure of the film and the high degree of inhibition even at the lowest concentration, the inhibition by DCNP was dose dependent (63, 66, and 78% inhibition at 1, 10, and 100 μM, respectively).

Photolabeling of Recombinant Sulfotransferases HAST1, HAST3, and HAST4—Three recombinant human liver STs, HAST1, -3, and -4, expressed in COS-7 cells were tested for their ability to photoincorporate 2-azido-[32P]PAP. Fig. 4 shows the photolabeling of the recombinant enzymes with 10 μM probe. Human liver cytosol labeled under the same conditions has been included for comparison (Fig. 4, lanes 1–3). With each recombinant enzyme, the major photolabeled protein band detected by autoradiography (Fig. 4, lanes 8, 10, and 12) corresponded to the major Coomassie Blue-stained protein seen on the gel. Furthermore, the labeling was completely inhibited in the presence of 50 μM PAP (Fig. 4, lanes 3, 9, 11, 13). No labeling was detected with control, non-transfected COS-7 cell homogenates (Fig. 4, lanes 4–6).

Photoaffinity Labeling of Human DHEA-ST and EST Expressed in E. coli—The preparations used in these experiments were human DHEA-ST and EST expressed in E. coli and partially purified by DEAE chromatography as described previously (14, 21). The results are shown in Fig. 5, and, as with the previous figure, photolabeling of human liver cytosol has been included for comparison (Fig. 5, lanes 1–3). Exposure of the recombinant proteins to UV light in the presence of 2-azido-[32P]PAP resulted in the labeling of proteins with approximate molecular masses of 36 kDa for DHEA-ST (Fig. 5, lane 4) and 31 and 36 kDa for EST (Fig. 5, lane 6). The specificity of the photolabeling was demonstrated by the high degree of inhibition of photoaffinity labeling in the presence of unlabeled PAP (Fig. 5, lanes 5 and 7).

Western Blot Analysis—To verify that 31–36-kDa proteins are photolabeled with 2-azido-[32P]PAP in both expression systems were indeed sulfotransferases, antibodies known to recognize STs were used for Western blot analyses of photolabeled COS-7 and E. coli-expressed preparations. The proteins from both expression systems were photolabeled, separated by SDS-PAGE, and electroblotted to nitrocellulose membrane. The blots were subjected to autoradiography followed by immunodetection with specific antibody. Human STs expressed in COS-7 cells were detected with a specific rabbit anti-human PST antibody. For the identification of the human cytosolic STs and E. coli-expressed EST, anti-human EST antibody was used (21), and for E. coli-expressed DHEA-ST, DHEA-specific antibody was used (17). The results are shown in Fig. 6 (COS-7 cell-expressed HAST1, -3, and -4) and Fig. 7 (E. coli-expressed DHEA-ST and EST). Human liver cytosol has been included in each figure for comparison. As shown in Fig. 6, human cytosolic protein and HAST1, -3, and -4 proteins were recognized by the PST antibody (Fig. 6A), and the immunoreactive bands also photolabeled 2-azido-[32P]PAP (Fig. 6B, lanes 5, 7, and 9).

Protein detected in samples photolabeled in the presence of
unlabeled PAP (Fig. 6A, lanes 6, 8, and 10) did not photoincorporate significant amounts of the probe (Fig. 6B, lanes 6, 8, and 10). Further, no photoincorporation was observed in control, non-transfected COS-7 cells (Fig. 6, A and B, lanes 11–13). The same results were found with the E. coli-expressed proteins. Recombinant protein detected with its corresponding antibody on the Western blot (Fig. 7A) was found by autoradiography to be photolabeled when incubated in the absence of unlabeled nucleotide (Fig. 7B, lanes 4 and 6) and protected when unlabeled PAP was present (Fig. 7B, lanes 5 and 7).

**DISCUSSION**

Direct photoaffinity labeling of various proteins with adenine derivatives, such as ATP, ADP, and S-adenosyl-L-methionine, has been described previously (22, 23). Additionally, reports in the literature have suggested that direct affinity labeling with adenosine 3'-(32P)phosphate 5'-phosphosulfate can be used to label PAPS-binding proteins (3). The mechanism of photactivated purines and purine nucleosides postulates the involvement of the C-8 position of the purine ring system (23). Another possibility is that UV irradiation results in the formation of free radicals of aromatic amino acids, which scavenge the nucleotide. In this work, we have developed an approach to ST labeling using PAP containing a 2-azido function as a photoreactive group. This mechanism, which involves covalent binding of the azido groups into specific amino acids, uses different functional groups than the procedure described above and, thus, targets different areas of the protein. Therefore, it can be anticipated that different amino acids could become radiolabeled. Moreover, labeling with probes that contain 32P allows for a much higher level of sensitivity in detecting the photolabeled proteins due to the higher energy of the decay products. This feature is especially important when using radiolabeling for the purification and characterization of the recombinant proteins.

A first series of studies was used to determine whether the probe, 2-azido-[32P]PAP, could be used as an effective photoaffinity ligand for specific labeling of PAPS-binding proteins in the mixture of proteins from crude human cytosolic preparations. Additionally, photoaffinity labeling of recombinant STs from COS-7 cell and E. coli expression systems was examined. We were able to demonstrate that, under standard conditions, the 2-azido-[32P]PAP probe efficiently bound to cytosolic STs as well as to several recombinant proteins with a high specificity (Figs. 1, 4, and 5).

As the next step, optimal photolysis conditions were determined using COS-7 cell-expressed human liver HAST3. We demonstrated that incubation of HAST3 with 2-azido-[32P]PAP followed by UV irradiation resulted in the photolabeling of a single 34-kDa protein (Fig. 2). The binding of 2-azido-[32P]PAP was concentration dependent with half-maximal binding at approximately 7.5 μM. Additionally, the labeling was inhibited in a concentration-dependent fashion by cold PAP and PAPS with 50% inhibition at 6.0 μM and 5.8 μM, respectively. This protection is an obligatory feature for demonstrating the true specificity of the photoaffinity labeling process. Moreover, labeling of HAST3 after preincubation with DCNP, a known inhibitor of the enzyme (24, 25), resulted in a concentration-dependent inhibition of photoaffinity incorporation. The inhibition of labeling of ST by DCNP was in agreement with previously published studies on the effect of DCNP on direct photoaffinity labeling with [35S]PAPS as the affinity probe (4). The observation that DCNP, a cosubstrate directed inhibitor which interferes with catalysis of the HAST3 reaction, also strongly inhibited the covalent binding of 2-azido-[32P]PAP could be explained by the possibility that PAP and/or PAPS bind to both the cosubstrate and the PAP/PAPS binding sites. As has been
demonstrated previously (4), 4-nitrophenol, a model sulfate acceptor for the phenol-specific STs, was neither required for nor enhanced the labeling of human HAST3 with 2-azido-[32P]PAP. Indeed, at high concentrations of 4-nitrophenol, photolabeling was significantly inhibited. The significance of this observation is not clear at the present.

After determination of the optimal photolysis conditions, we designed experiments to determine whether 2-azido-[32P]PAP could also be used to photolabel other human STs expressed in different expression systems. Figs. 4 and 5 show the photoaffinity labeling of COS-7 and E. coli-expressed human STs. In both series of experiments, crude liver cytosol was photolabeled for comparison. These studies demonstrated that three human STs, HAST1, -3, and -4, were each expressed as one single protein with apparent molecular masses of 32, 34, and 32 kDa, respectively. It should be emphasized that, in our experience, only a fully expressed enzyme that possesses catalytic activity can be photolabeled. Therefore, the ability to photoincorporate the probe can be used as a criterion of successful expression. The above considerations can also be applied for the characterization of the bacteria-expressed proteins. Fig. 5 shows the photolabeling of two human cDNA-expressed steroid STs, DHEA-ST and EST, which were expressed as single proteins in an E. coli expression system. The labeling was UV-dependent, competitively inhibited by unlabeled PAP, and the labeled proteins were identified as STs by Western blot analysis using specific anti-DHEA-ST and EST antibodies.

Although progress in molecular biology has provided a better understanding of the overall genetic organization and expression of the STs, the amount of information available on the relationship between the structure and function of these enzymes is limited. STs require the activated sulfate donor PAPS as a cofactor. A putative nucleotide binding motif termed the P-loop, found in ATP- and GTP-binding proteins (27), and the authors suggested that this consensus sequence might constitute the PAPS binding site. It was recently confirmed by Komatsu et al. (28) that the P-loop, highly conserved in all STs, is required, at least in part, for binding of the activated sulfate donor. In different studies, Falany et al. (29), using site-directed mutagenesis techniques, investigated the suggestions from previous experiments (16) that a cysteine residue might be located near the PAPS binding site of the STs. Bacterial expression of human p-PST with the cysteine at position 70 converted to serine indicated that the cysteine was essential for activity or substrate binding. However, the mutant enzyme is significantly more sensitive to thermal inactivation.

Recently, Zheng et al. (30) investigated the PAPS binding site using ATP dialdehyde as an active site-directed affinity label for the PAPS binding site of rat aryl ST IV. It was demonstrated that the affinity label was bound to a hexapeptide at both lysine 65 and cysteine 66. These affinity-labeled amino acids are located within a region in the sequence of AST IV that shows considerable homology with various STs possessing diverse specificities for acceptor substrates.

In this paper, we have demonstrated that 2-azido-[32P]PAP can be used as a photoaffinity probe to covalently and specifically tag PAP-binding proteins in cytosolic and membrane fractions of rat and human liver and recombinant ST proteins. Investigations of the actual site of this linkage of protein and radioactive probe are in progress in our laboratory.