Purification and characterization of recombinant hydroxysteroid sulfotransferase

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Abstract

Sulfotransferases are important enzymes involved in the conjugation of many foreign chemicals and endogenous substances. The goal of work outlined in this paper was to heterologously express five different forms of this family of enzymes cloned from human and rat liver in bacteria. A second goal was to develop methods for the purification of these enzymes from bacterial cytosol. Expression of several forms of these enzymes was complicated by their deposition in bacterial inclusion bodies. Rules governing the deposition of sulfotransferases in inclusion bodies could not be developed on the basis of their secondary structures or enzymatic activities.

Introduction

Sulfonation is an ubiquitous pathway in biology and is critical for the metabolism of many hormones and neurotransmitters as well as a range of foreign compounds. This important pathway of metabolism is catalyzed by an emerging gene superfamily of cytosolic sulfotransferases (STs) (Figure 1). Within the past 5 years, cDNAs for many of these enzymes have been cloned and characterized. Current work in our laboratory is directed at exploring the involvement of several isoforms of hydroxysteroid sulfotransferase (HST_{20}, HST_{40}, and HST_{60}) in the metabolism of steroids. We have subcloned cDNA for the rat forms of these enzymes into high efficiency expression vectors and have expressed these forms in several strains of *E. Coli*. The purpose of this work is to purify and characterize mammalian recombinant forms of HST expressed in bacteria.

Sulfotransferase (STs) are classified as phase II conjugate enzymes which detoxify compounds by the addition of a sulfuryl group from 3 phospoadenosyl -5 phosphosulfate (PAPS). This addition makes a compound more acidic, and more water soluble which facilitates it's excretion and inactivation.

Activities of STs are found in extrahepatic tissues such as brain, intestine and kidney with a varying specificity for different substrates. Sulfotransferase's regulating estrogen, testosterone, and other steroids, vary between sexes. Other genetic

polymorphism[s] change an organism's ability to metabolize many xenobiotics, as well as endogenous substances such as steroids.

Methods

Expression vector

Constructs of three isoforms (HST_{20}, HST_{40}, and HST_{60}) of hydroxysteroid sulfotransferase in expression vectors were generously provided by Dr. Hansruedi Glatt of the German Institute for Nutritional Research in Potsdam, Germany. HST_{20} was subcloned from the cloning vector, pUC18, into the expression vector, pET 17b. This construct was used to express the enzyme in *E. coli*., BL21(DE3) pLyss. ST-40 and ST-60 in the expression vector pKK233-2 were used directly for expression experiments in various strains of *E. coli*., JM105 and BL21(DE3) pLyss.

Culturing bacteria

E. coli. transfected with the above vector, and stored as glycerol stocks, were grown in 2 ml LB (Lucia - Bectani) broth pH 7.4 with Ampicillin and Chloroamphenicol. The bacteria were incubated overnight at 30 degrees at 220 rpm in an Innova 4000 incubator shaker. 250 ul of this solution was transferred to a 500 ml of LB broth, containing the selection antibiotics in Erlenmeyer flask and incubated for approximately 5 hours to an optical density (O.D.) of .6 at 600 nm. The recombinant gene was induced with 1mM IPTG and the mixture was incubated overnight at 30 degrees at 220 rpm. The cells were pelleted and frozen at -70 degrees until harvesting.

Harvesting and extracting bacteria for induced protein

The Bacterial pellets were frozen at -70 degrees for at least 30 minutes and thawed quickly to break bacterial cell walls. The pellet was resuspended in 20 ml 40mM Tris HCl, pH 8.0, containing 5mM EDTA (Lysing Buffer). In bacteria not engineered to express lysozyme (e.g. E.Coli, BL21(DE3)), 500 ul of freshly prepared lysozyme (10 mg/ml) was added and incubated at room temperature for 15 minutes. Then 500 ul of 5% Brij-58 containing 200 mM $MgSO_4$ was added and incubated for 15 minutes at room temperature. Bacterial extracts were sonicated at a high setting on an Ultrasonic Homogenizer 4710 series sonicator, and subsequently centrifuged at 12,000 x g to sediment inclusion bodies and cell debris. Some cells were broken by preequilibrating cell suspension at 3000 psi in a nitrogen bomb before extraction. The supernatant was finally centrifuged at 100,000 x g in a Beckman model M-80 ultracentrifuge to obtain bacterial cytosol used for enzyme assays.

Columns

Multiple columns such as the Mono Q and High Trap Q anion exchange columns from Pharmacia were used. The final purification step was performed with an ADP affinity column.

Sulfotransferase Activity

Sulfotransferase activity was measured with radioactivity using either [^{3}H]-labeled steroids or [^{35}S]-labeled PAPS. Incubations with [^{3}H]-steroids were performed by incubating aliquots of bacterial cytosol or purified fractions in 200 ul of .05 M Tris-HCl buffer, containing: 10 mM $MgCl_2$, 1mM DTT and 5 uM [^{3}H]-DHEA or [^{3}H]Pregnenolone. Kinetic experiments were performed by varying the concentrations of [^{3}H]-labeled steroids between 0.50 and 100 uM.

Assays with ^{35}S-PAPS were performed in 100ul of the same buffer described above and unlabeled steroids or para-nitrophenol as indicated in the legend to Figure and Tables.

Protein was assayed using the Lowery procedure with bovine serum albumin as standard.

SDS-PAGE

The purity of the sulfotransferase was determined with using 1.5mm. 10% SDS PAGE mini gel. The lanes was loaded with 25 ug, 10 ug, or 1 ug in a standard loading buffer. The gel was run at 130 volts for approximately 45 min. The protein bands were stained with coomassie blue or determined through Western Blotting.

Western Blotting

The protein from the mini gel was transferred to nitrocellulose at 50 volts for 2 hours in transfer buffer. The nitrocellulose membrane was washed in TBST for 5 minutes followed by 5% BSA in TBST for 60 minutes. A primary polyclonal antibody prepared in rabbit against hDHEA-ST (1:1000) dilution in the BSA solution was incubated overnight. The following day, The membrane was washed six times with TBST for 5 minutes each wash before incubation with 5% BSA and a 1:5000 dilution of secondary antibody (Horse-radish peroxidase anti-rabbit from donkey). The membrane was then washed 6 times with TBST for 5 minutes each wash. The nitrocellulose was developed using ECL developer and exposed to x-ray film.

Discussion

With expression of sulfotransferase in *E. coli*., bacterial response causes the aggregation of enzyme in particulate matter known as inclusion bodies. Studies of structural differences between HST_{20}, HST_{40}, HST_{60}, and hDHEA reveals only 0.4 % differences in secondary structure between all of these forms. Human sulfotransferase is 55% homologous to rat sulfotransferase, yet only a 0.4% difference in the secondary structure of these proteins is predicted according to the rules suggested by Rost et al. (1993). Thus differences based on primary and secondary protein structure do not give a clear understanding of which protein initiates a response to form inclusion bodies.

Of the five enzymes studied (HST_{20}), HST_{40}, HST_{60}, hDHEA, and AST IV), the HST_{20} and HST_{40} were initially trapped in inclusion bodies in E.coli., while HST_{60} and hDHEA were in cytosolic fraction. In Salmonella, HST_{20} and HST_{40} were in cytosolic fraction, while HST_{60} and hDHEA were trapped in inclusion bodies. The AST IV enzyme, a phenol sulfotransferase, was seen in the cytosolic fraction in both *E. coli* and Salmonella fractions. One clone of HST_{20} in E.coli produced cytosolic sulfotransferase. These data suggest that inherent properties of bacterial expression strains may also determine the fate of expressed proteins. Factors such as chaperonins and their role in protein folding to native and denatured forms during overexpression and heat shock conditions may be related to inclusion bodies.

Conclusion

- Multiple forms of Rat and Human Sulfotransferase can be readily expressed in bacterial cytosol and purified.
- Overexpression of some forms of sulfotransferases stimulates the formation of inclusion bodies. The formation can not be related to primary or secondary protein structure and is not always consistent, e.g., HST_{20} is expressed in inclusion bodies of some clones of *E.Coli* and in cytosol of other clones of the same bacterial strain.
- Mechanisms underlying the formation of inclusion bodies remain ill defined and variable.

References

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Table 1. Summary of purification of rat liver hydroxysteroid sulfotransferase (HST_{20}) expressed in *E.Coli*. inclusion[1] bodies

Purification Step[2]	Activity (DPM)	Total Activity (pmole/min)	Protein Conc. (mg/ml)	Specific Activity (pmole/min/mg)
Stock ST-20	2171	6555	5.43	60.37
Nitrogen Bomb	2770	8360	7.68	52.29
Sonicate Foam	3890	11740	6.86	83.18
Sonicate	2744	11299	6.95	57.22
Centrifuge 14,000 rpm	3566	10763	6.02	89.40
Centrifuge 100,000 x g	3342	10086	5.91	85.34
Storage 1 week (-70 °C), Centrifuge 100,000 x g	8209	17791	8.63	143.55
DHEA-Sepherose Frac#23[3]	2801	2115	0.92	460.00

Notes:
1)Inclusion bodies form as a result of improper binding or folding of a protein; their formation protects the organism from foreign, potentially dangerous protein. When

trying to express our sulfotransferase enzyme in vitro, the bacterial vector "locked" our proteins in this unfolded conformation.

2)Through the multiple steps, the enzyme is purified from its bacterial contents; improved purification is indicated by the increase in specific activity (last column).

3)Separation was carried out with a DEAE-Sephedex column (an anion exchange column) eluted with 1M NaCl gradient. Two peaks were observed: The first peak was the wash out fraction from overloading the column; the second peak was our ST20 enzyme which was purified 7.5 fold in comparison with the initial specific activity (pmoles/min/mg)

Table 2. Purification of HST_{40} in cytosol of *E. Coli* jm105 after disruption of inclusion bodies at 3000 psi in a nitrogen bomb

Purification Step	Volume (ul)	Total Activity (pmole/min/sample)	Protein Conc. (mg/ml)	Specific Activity pmole/min/mg)	Percent Recovery
Ammonium Sulfate	200	593.0	6.82	434.0	100.0
Superose 6B	15000	273.0	--	--	46.0
Concentrated Fraction	910	311.0	1.40	244.0	52.4
Stored (4 days)	910	362.0	1.40	284.0	61.0
Mono Q Column	2000	126.0	--	--	0.3
Concentrated Mono Q	100	82.5	2.46	333.5	13.9

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Table 3. Summar (HST_{20}) expressed in *E.-Coli* cytosol

Purification Step	Volume (ml)	Total Activity (pmole/min/sample)	Protein Conc. (mg/ml)	Specific Activity (pmole/min/mg)	Percent Recovery
Stock	5.00	99480	15.76	1262	100.0
Ammonium Sulfate	1.90	27772	18.60	776	27.9
Predialysis	3.50	53921	11.53	1336	54.2
Dialysis	6.86	83672	7.51	1624	98.1
High Trap Q (1of 6.86ml)	2.51	16827	1.06	6312	116.0
ADP affinity column	0.30	3899	0.14	91525	43.5

Figure 1. The process of sulfonation is critical for the metabolism of many chemical substances.

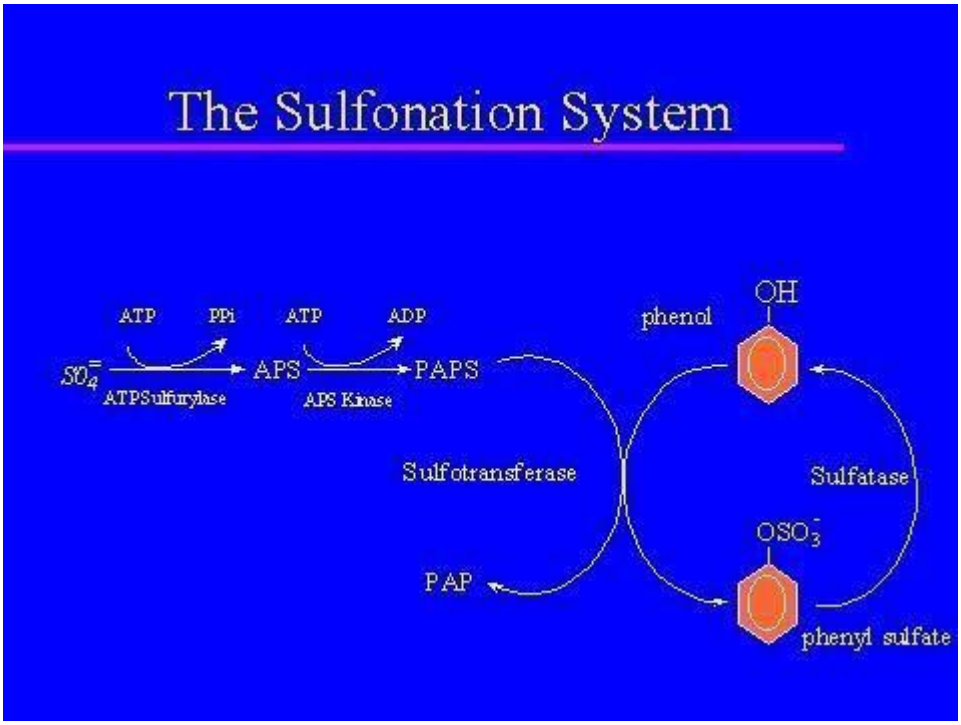


Figure 2.

The Mono-Q column is an anion exchange column that separates HST_{40} from other proteins having different electrical charges. HST_{40} is eluted with 1M NaCl in 0.02 M Tris.HCl, pH 7.4 containing 0.05 mM dithiothreitol (DTT).The cumulative recovery of the NaCl is shown as solid squares. The emergence from the column of ^{3}H-labeled DHEA is monitored by measuring the activity of the eluted fractions (triangles). The highest activity is seen at fraction #8. Separation from other proteins is monitored by measurements of optical density (O.D.) at 280 nm (diamonds).

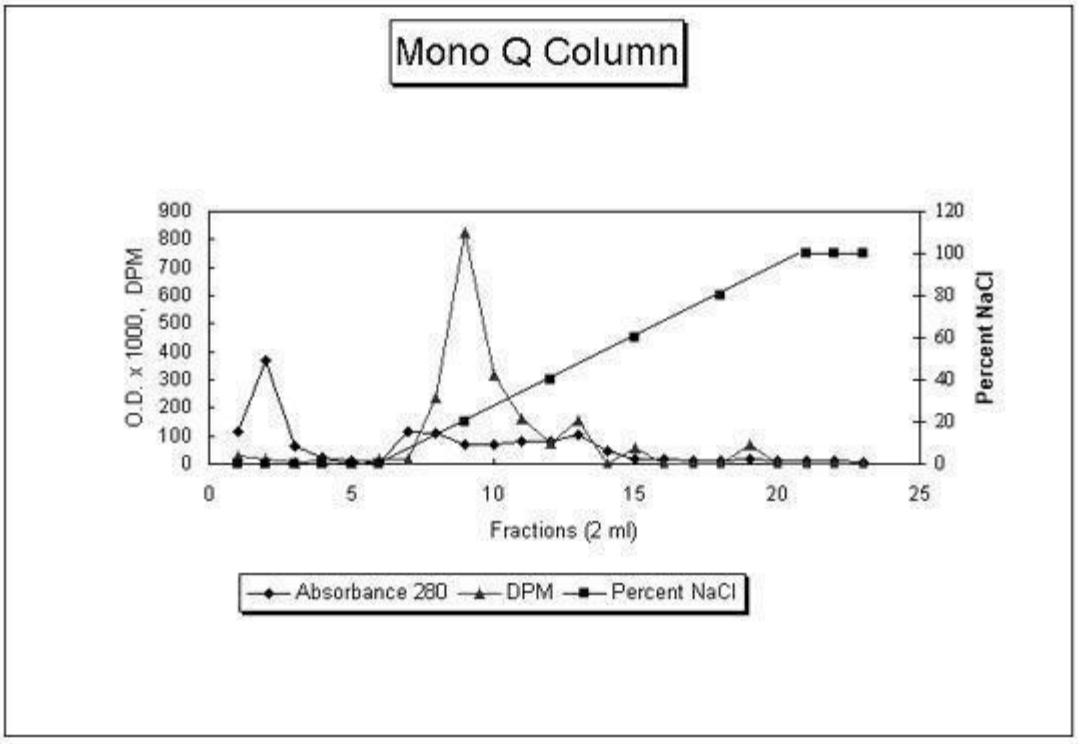
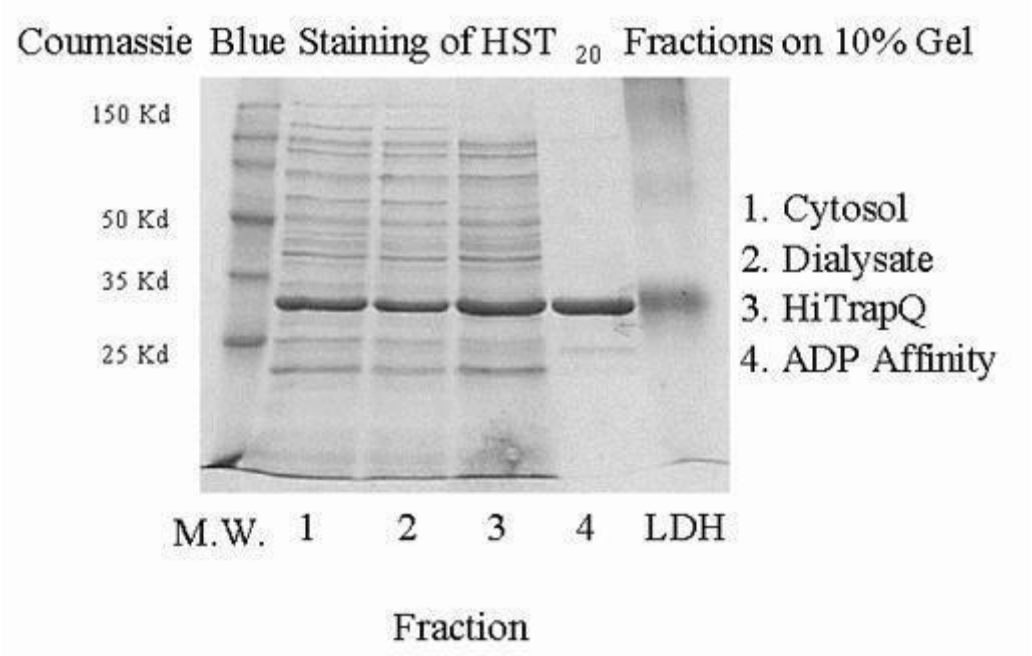


Figure 3. Coumassie blue staining of purified fractions of sulfotransferases separated on a 10% polyacrylamide gel



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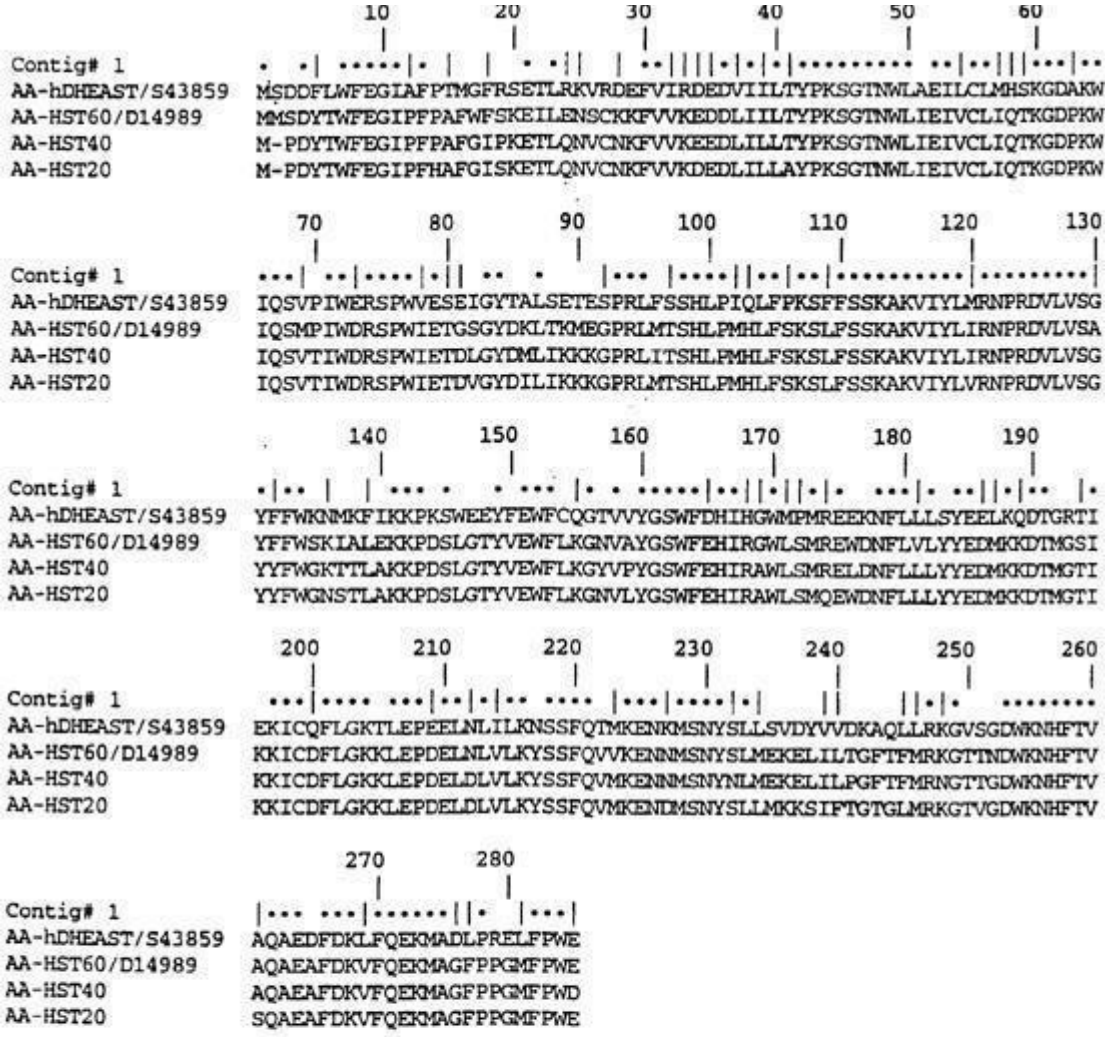
igure 4. Alignment and Predicted Secondary Structures of HSTs Cloned from Rat and Human cDNA Libraries



	Alpha Helix	Beta Sheets	Loop
HST-20	**43.0%**	**13.0%**	**44.0%**
HST-40	**43.0%**	**12.7%**	**44.4%**
HST-60	**42.8%**	**13.0%**	**44.2%**
HDHEA	**43.5%**	**12.6%**	**43.9%**