Characterization of the active site and coenzyme binding pocket of the monomeric UDP-galactose 4'-epimerase of Aeromonas hydrophila

Shivani Agarwal1, Neeraj Mishra2,4,*, Shivangi Agarwal3,4, # & Aparna Dixit1,*

1Gene Regulation Laboratory, 2Biophysical Chemistry Laboratory, 3Laboratory of Molecular Biology and Genetic Engineering, School of Biotechnology, Jawaharlal Nehru University, New Delhi-110067, India, 4Department of Biological Chemistry, Weizmann Institute of Science, Rehovot-76100, Israel

Aeromonas hydrophila is a bacterial pathogen that infects a large number of eukaryotes, including humans. The UDP-galactose 4'-epimerase (GalE) catalyzes the interconversion of UDP-galactose to UDP-glucose and plays a key role in lipopolysaccharide biosynthesis. This makes it an important virulence determinant, and therefore a potential drug target. Our earlier studies revealed that unlike other GalEs, GalE of A. hydrophila exists as a monomer. This uniqueness necessitated elucidation of its structure and active site. Chemical modification of the 6xHis-rGalE demonstrated the role of histidine residue in catalysis and that it did not constitute the substrate binding pocket. Loss of the 6xHis-rGalE activity and coenzyme fluorescence with thiol modifying reagents established the role of two distinct vicinal thiols in catalysis. Chemical modification studies revealed arginine to be essential for catalysis. Site-directed mutagenesis indicated Tyr149 and Lys153 to be involved in catalysis. Use of glycerol as a cosolvent enhanced the GalE thermostability significantly. [BMB reports 2010; 43(6): 419-426]

INTRODUCTION

Galactose utilization in majority of gram-negative bacteria occurs by the Leloir pathway (1). UDP-galactose 4'-epimerase (GalE), a pyridine nucleotide-dependent oxidoreductase, catalyzes the interconversion of UDP-galactose and UDP-glucose. The UDP-galactose donates its galactose moiety to lipopolysaccharide (LPS) precursors for core biosynthesis. The galE mutants synthesize LPS deficient cell wall and display attenuated virulence (2). The active site of epimerase is divided into three regions; substrate binding, coenzyme binding and catalytic region. The hexose moiety of the nucleotide sugar and the pyridine moiety of the coenzyme are located adjacent to each other at the catalytic site for hydride transfer and epimerization (3). Although considerable work has been done on the reaction pathway and the reaction mechanism; little is known about the architecture of the active site of GalEs except for few reports on yeast epimerases (4-6).

Classical dehydrogenases exploit a histidine residue that functions as a general base in initiating enzyme catalysis (4). Chemical modification studies with Kluyveromyces fragilis enzyme revealed the requirement of an essential thiol, histidine and arginine residues in the overall catalytic process (4-6). Aeromonas hydrophila is reported to infect both humans and fishes, and results in major economic losses to aquaculture industries (2). GalE mutants of A. hydrophila have been reported to be avirulent and therefore it can be used as a potential drug target. Amino acid sequence analysis of the A. hydrophila GalE revealed a characteristic Tyr-X-X-X-Lys couple, at 128-133rd position, a key player in catalysis (7). However, the GalE of A. hydrophila exhibits interspecies variation and heterogeneity at its structural and functional level and therefore, the differences between the GalE of the host and the pathogen can be exploited for drug designing (8). Therefore, the present investigation was carried out to identify the crucial residues involved in catalysis and cofactor binding, their localization and organization within the active site of the exception-ally unique monomeric GalE of A. hydrophila.

RESULTS AND DISCUSSION

Histidine residue is crucial for the activity of the 6xHis-rGalE

The classical catalytic mechanism of several dehydrogenases is initiated by a base, usually a histidine leading to the formation of enzyme-bound intermediates (4). In view of this paradigm, it was pertinent to explore the presence of any histidine residue located in the vicinity of the catalytic region of the active site. Incubation of the 6xHis-rGalE (recombinant 6xHis tag protein) with increasing concentrations of DEPC resulted in a time-dependent loss of activity (Fig. 1A). The control/unmodified
6xHis-rGalE in 1% ethanol was active during the incubation obviating the possibility of the loss of activity due to organic solvent. Plot of log remaining activity against time \((1-e^{-kt})/k\) for inactivation at various concentrations of the reagent yielded a straight line (Fig. 1B). The increase in \(A_{246}\) with increasing concentration of the modifier demonstrated a dose dependent modification (Fig. 1B, inset). The reaction order (one) obtained for the enzyme inactivation, with respect to the DEPC concentration, suggested that approximately one molecule of DEPC-inactivated one molecule of the 6xHis-rGalE. This is in agreement with the earlier observations on the inactivation of *K. iragilis* GalE (4).

DEPC is known to react with residues other than histidine like tyrosine, tryptophan and cysteine (4). The intensity and nature of protein fluorescence spectrum of the 6xHis-rGalE remained unaffected upon DEPC modification thereby ruling out the possibility of either tryptophan or tyrosine modification (Fig. 1C). The potential of hydroxylamine to remove the carbethoxy group exclusively from DEPC-modified histidines, and not from lysine/cysteine was exploited for the reversal experiments. A substantial (80%) recovery of the DEPC-modified 6xHis-rGalE activity with hydroxylamine suggested that the modified amino acid is histidine (Fig. 1D).

Previous studies have demonstrated that the DEPC results in

![Fig. 1. Histidine is crucial for the 6xHis-rGalE activity. (A) Kinetics of inactivation of the 6xHis-rGalE by DEPC. (B) A plot of log of pseudo-first order rate constant for inactivation \((k_{obs})\) at varying DEPC concentrations Vs log [DEPC]. Inset depicts \(\Delta A_{246}\) of the DEPC-modified 6xHis-rGalE (C) Relationships between DEPC-induced inactivation of the 6xHis-rGalE with the protein fluorescence (a), coenzyme fluorescence (b), efficiency of energy transfer (c), and activity (d) are plotted against the incubation time. (D) Percentage residual activity of DEPC-modified and reactivated 6xHis-rGalE with hydroxylamine (E) DEPC modification did not alter the adenine moiety of the 6xHis-rGalE-bound NAD\(^+\). The graph represents % activity of NAD\(^+\) in the native and DEPC-modified 6xHis-rGalE; with rPGI as positive control and glucose-6-phosphate dehydrogenase without the rPGI as negative control. (F) Reduction of NAD\(^+\) in the DEPC-modified enzyme. The coenzyme fluorescence of the 6xHis-rGalE, 6xHis-rGalE with 5'-UMP and NaBH\(_4\), modified 6xHis-rGalE+5'-UMP+NaBH\(_4\) were recorded.](http://bmbreports.org)
the ring opening of the imidazole moiety of adenosine (9). However, treatment of \textit{A. hydrophila} 6xHis-rGalE with DEPC had no effect on the adenine moiety of bound NAD$^+$. About 75% of the NAD$^+$ was active in the modified enzyme which was almost comparable with the unmodified enzyme (90%), thus suggesting that both the enzymes were able to reduce NAD$^+$ efficiently (Fig. 1E). The data thus suggest that the inactivation of the 6xHis-rGalE upon DEPC treatment was not due to any non-specific modification of the coenzyme’s adenine moiety, and was solely due to the histidine modification.

Failure of the substrate (UDP-galactose) to afford protection to the histidine against DEPC-induced inactivation provided a clue that the histidine is not located at the substrate binding region of the active site (data not shown). Further evidence in this direction was obtained by assessing the fluorescence energy transfer (FRET) phenomenon. The characteristic fluorescence of the bound coenzyme was exploited to monitor subtle changes at the active site of the enzyme. It has been observed that a small amount of protein energy fluorescence was transferred to the coenzyme fluorophore of the 6xHis-rGalE and was emitted at a higher wavelength with two emission maxima (340 nm, protein fluorescence; 450 nm, coenzyme fluorescence) (data not shown). Fairly good kinetic correlation was observed between the loss of enzyme activity upon modification of histidine and loss of energy transfer process (Fig. 1C). This abolition of the energy transfer upon DEPC modification with no loss of enzyme and coenzyme fluorescence suggested a conformational change in the vicinity of the coenzyme binding region, thus possibly leading to the improper orientation of the two fluorophores.

Epimerases are reductively inactivated by NaBH$_4$ but the reduction is sluggish in the absence of UMP (4). An attempt was made to reduce the bound NAD$^+$ of the native and DEPC-modified 6xHis-rGalE by NaBH$_4$ to reinforce its presence at the coenzyme binding site. The NaBH$_4$ reduction of the 6xHis-rGalE led to enhanced fluorescence due to the formation of catalytically abortive enzyme-NADH complex, which is stabilized by 5’-UMP. However, after DEPC modification, the coenzyme (NAD$^+$) of the 6xHis-rGalE was not amenable to chemical reduction (Fig. 1F). The data thus demonstrated that DEPC modification rendered the coenzyme completely resistant to NaBH$_4$ treatment.

Collectively, the results revealed that the histidine is located in the close proximity to the coenzyme binding region and not in the substrate binding pocket.

**Role of cationic residues in enzyme catalysis**

It has been reported that an arginine is located in the substrate binding region of \textit{K. fragilis} GalE (5). The guanido-nitrogen of arginine at 292$^{nd}$ position in the GalE of \textit{E. coli} forms a bond with the oxyanion of $\alpha$-phosphate of its substrate (5). Interestingly, arginine residues (R231, 291 and 292) are conserved amongst species (7). We have earlier reported that the fluctuations in the pH drastically affected the 6xHis-rGalE conformation and its activity. We thus inferred that the enzyme attained a relatively more ordered structure at higher pH, and further established the role of electrostatic interactions in maintaining its nativity (8). The 6xHis-rGalE substrate, UDP-galactose, being negatively charged at physiological pH, prompted us to investigate the possible involvement of positively charged residue(s) at the active site. This was confirmed by observing a progressive loss of 6xHis-rGalE activity in a dose-dependent manner, upon modification with 2,3-cyclobutanediene (Fig. 2A). However, the inactivation did not follow monophasic pseudo-first order kinetics, rather displayed biphasic kinetics, suggesting that few arginine residue(s) are much more accessible to the reagent.

**Functional significance of two distinct active site thiols**

In classical dehydrogenases, the hydride transfer is facilitated either by a metal ion e.g. in yeast alcohol dehydrogenase or by an amino acid which acts as a general base as in lactate dehydrogenase (10, 11). The lack of evidence in support of epimerases being metalloenzymes, we speculated that the thiol of epimerase either alone or in tandem with histidine may facilitate hydride transfer. This is analogous with the glyceraldehyde-3-phosphate dehydrogenase where the thiol in combination with the pyridine ring generates charge-transfer complex (12). S-adenosylhomocysteinase and TDP-glucose oxidoreductase are other examples of oxidoreductases involving cysteine and histidine residues for catalysis (3, 13).

\textit{A. hydrophila} 6xHis-rGalE was inactivated by increasing concentrations of DTNB (Fig. 2B). An increase in $A_{412}$ suggested specific modification of the thiol groups (Fig. 2B, inset). The inactivated enzyme was partially reactivated by DTT (Fig. 2E). Also, progressive and coordinated loss of coenzyme fluorescence induced by DTNB-inactivation was restored partially by DTT (Fig. 2C). Further insight into the role of two thiols was obtained with another thiol modifying agent, NEM. Concurrent with the results obtained with the DTNB modification, NEM indicated the crucial role of thiol residues in catalysis (Fig. 2E). However, a very striking and substantial difference in the modification pattern was observed with the two reagents. DTNB generated inactive and non-fluorescent enzyme (dark enzyme) (Fig. 2C) while NEM led to the formation of inactive, but fluorescent enzyme molecules. The loss of coenzyme fluorescence in the NEM-modified 6xHis-rGalE upon DTNB addition could therefore be attributed to the simultaneous modification of an additional thiol residue other than the essential one (Fig. 2D). The second additional thiol in discussion therefore might not be accessible to modification by NEM, but lost its integrity upon DTNB addition. This is indicative of the fact that DTNB modified a second thiol in the vicinity of the pyridine fluorophore, which subsequently resulted in quenching of the coenzyme fluorescence (Fig. 2D).

It is known that unlike NEM-induced modification, DTNB modification is reversible. To prove the discriminatory and
non identical roles of two thiols beyond doubt, protection experiments employing the strategy of consecutive modification with both the reagents were performed. In the first set, the enzyme was inactivated with DTNB followed by NEM. This could have two possible results: If both the reagents modified the same thiol, the incubation with DTNB will protect the second thiol from NEM modification. Therefore, reactivation with DTT should restore the activity partially. On the other hand, if both the reagents are modifying two distinct thiols, inactivation with DTNB will fail to afford any protection against subsequent irreversible modification by NEM. Therefore, in this case, no regeneration of the enzyme activity is expected. Fig. 2E depicts the activity of the 6xHis-rGalE, subjected to different treatments. The sets I-V depict the potential of the modifying agents (DTNB and NEM) in the inactivation and reactivation (DTT) compared to the untreated enzyme. The 6xHis-rGalE completely lost its activity when both DTNB and NEM were added together (Set VI). Set VII shows that the enzyme regained 40% of its original activity by DTT after modification with DTNB and NEM. This suggests that the loss of enzyme activity was due to the modification of a unique thiol residue. Therefore, the study revealed the existence of a non-catalytic second thiol, resistant to NEM modification, involved primarily in generating and maintaining the coenzyme structure.

Studies conducted for *K. fragilis* also demonstrated similar mechanism of catalysis involving two distinct thiol residues with entirely different functions (5), thereby suggesting that even though *A. hydrophila* epimerase has a unique quaternary structure.
and subunit composition, it follows similar epimerization kinetics. Crystallization of the 6xHis-rGalE will provide a definitive and direct evidence for the role of essential thiols, arginine or histidine in catalysis.

**Catalytic parameters of the 6xHis-rGalE mutants of *A. hydrophila***

Chemical modification studies provided an insight into the role of histidine, thiol and arginine residues near the active site of the enzyme. To delineate the active site residues of the 6xHis-rGalE, and to decipher the catalytic mechanism, site-specific mutants were generated. The 6xHis-rGalE mutants, Y149G, K153N and Y149G-K153N were expressed (Fig. 3A, lanes 2, 5 and 8, respectively) and purified (lanes 3, 6 and 9, respectively). The kinetic coefficients, $K_m$ and $V_{max}$, were derived using Lineweaver-Burk plot (Fig. 3D). The enzyme inactivation after substitution of the highly conserved Lys153 and Tyr149 indicated an essential role of these residues in catalysis. Crystal structure of the *E. coli* GalE revealed that the 2'- and 3'-hydroxyl groups of the nicotinamide ribose of the coenzyme formed hydrogen bonds with the ε-NH$_2$ group of lys153 (14). The substitution of lysine to glutamine at 153$^{th}$ position in the 6xHis-rGalE affected the catalytic activity only slightly; however, the NAD$^+$ binding potential was reduced dramatically, thus substantiating the role of this residue in coenzyme binding (Fig. 3B).

It has been known that the epimerization involves a general acid-base that facilitates hydride transfer from galactosyl or glucosyl substrates to the nicotinamide of NAD$^+$. The close proximity between the nicotinamide ring of NAD$^+$ and the phenolic oxygen of Tyr149 in the GalE of *E. coli* suggests that it might participate in the charge-transfer phenomenon (14). In agreement to this, the *A. hydrophila* 6xHis-GalE with Tyr149 mutation was completely inactive, thereby suggesting the role of Tyr149 in general acid-base catalysis (Fig. 3D). The decrease in activity could therefore be attributed to the unfavorable

<table>
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<th>Enzyme</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (μmoles/mg/min)</th>
<th>$K_{cat}$ (S$^{-1}$)</th>
<th>$K_{cat}/V_{max}$ (m$^{-1}$S)</th>
<th>NAD$^+$ binding</th>
<th>$T_m$ (°C)</th>
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<td>116</td>
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<td>Y149G</td>
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<tr>
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<td>46</td>
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folding and rigidity of the enzyme (as inferred from slightly increased $T_m$) as well as loss of -OH group. Also, tyrosine is solvent exposed and its substitution with an uncharged small glycine possibly results in the burial of the active site, making it slightly inaccessible to the substrate, and thus affecting the hydrophobic interactions significantly. The double mutant did not display any activity even at higher substrate and enzyme concentrations. Further, elucidation of the crystal structure of the wild type and mutant enzymes would aid in better understanding of the mechanism(s) underlying catalysis.

The fluorescence and far UV-CD spectra (Fig. 3B inset and 3C, respectively) of the wild type 6xHis-rGalE and its mutants were comparable, suggesting that the point mutation did not cause substantial unfolding (tertiary structure) or other gross significant perturbations in the secondary structure.

Thermal denaturation analysis of the wild type 6xHis-rGalE and its mutants revealed an increase in $T_m$ ($\approx 2^\circ$C) of the Y149G mutant with respect to the wild type and K153N mutant (Fig. 3D). A slight decrease in $T_m$ ($1^\circ$C) upon substitution of lysine with glutamine at 153rd position in the 6xHis-rGalE could be a result of the impaired NAD$^+$ binding, which probably resulted in the perturbation of the electrostatic interactions and loss of hydrogen bonds.

**Effect of glycerol on the 6xHis-rGalE stability**

The potential of glycerol in providing protein(s) with protection against denaturation has been demonstrated (15). This has led to its usage as a cryoprotectant in order to improve the long-term storage of biopharmaceuticals. Considering the potential of *A. hydophila* GalE as a drug target, stability of the protein in context to its shelf-life is an important parameter. Therefore, the effect of glycerol on protection of the 6xHis-rGalE against heat stress was investigated. The data obtained from UV-melting curves demonstrated an increase in the $T_m$ from 48.5-57$^\circ$C with increasing concentrations of glycerol (Fig. 4A). The residual activity was also determined after denaturing the enzyme in the presence or absence of glycerol. Fig. 4B shows that the enzyme in 50% glycerol could retain 90% of its activity, whereas only 20% activity was observed for the identically treated control enzyme (Fig. 4B). The data therefore suggested that glycerol protected the enzyme against heat-induced unfolding, enhanced its structural stability, and reduced its rate of inactivation. The calorimetric studies depicted a shift in the $T_m$ of the 6xHis-rGalE by 8-9$^\circ$C in 50% glycerol (Fig. 4C). The results from spectroscopy and calorimetry corroborated with each other, and suggested that the protective effect of glycerol was due to the enhanced structural stability of the 6xHis-rGalE. The fluorescence spectra of the 6xHis-rGalE de-

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**Fig. 4.** Glycerol enhances the thermostability of the 6xHis-rGalE. (A) Effect of glycerol on heat inactivation of the 6xHis-rGalE. (B, C) The $T_m$ of the 6xHis-rGalE in the presence of different concentration of glycerol using (B) UV-absorption spectroscopy and (C) differential scanning calorimetry. The intrinsic fluorescence (D) and CD spectra (E) of the 6xHis-rGalE after heat inactivation in the presence and absence of glycerol. CD spectrum of the guanidine-hydrochloride treated 6xHis-rGalE was included as control.
nated in the presence of glycerol showed a blue shift in the emission maxima (357-327 nm) in comparison to the control denatured 6xHis-rGalE, suggesting formation of a compact globular structure in glycerol that could protect the 6xHis-rGalE from thermal denaturation. (Fig. 4D). The intrinsic fluorescence results were further substantiated by the CD spectra which revealed that 6xHis-rGalE containing glycerol could efficiently resist heat-induced changes in the secondary structure (Fig. 4E). Similar results were obtained with creatine kinase in glycerol, suggesting that the cosolvent shifts the equilibrium of the denatured enzyme towards the native state (16).

Thus, the role of catalytic residues in the GalE of A. hydrophila is illustrated. It is of interest to note that though the 6xHis-rGalE is unique as it exists as a monomer, residues near its active site exhibited properties similar to those of dimeric GalEs. Elucidation of the crystal structure would facilitate better understanding of its interactions with the substrate and coenzyme and also identify the regions that could be targeted for drug design.

MATERIALS AND METHODS

Materials

Diethylpyrocarbonate (DEPC), 5,5′-dithiobis-(2-nitrobenzoic acid) DTNB, 2,3 butanedione, N-ethylmaleimide (NEM), hydroxylamine, DTT and oligonucleotides were procured from Sigma Co., USA. Quick-change site-directed mutagenesis kit was from Stratagene, USA. E. coli, DH5α and BL21 (DE3) strains were procured from Novagen, USA.

Site-directed mutagenesis of the 6xHis-rGalE and purification of the 6xHis-rGalE and its mutants

Point mutants of A. hydrophila GalE were constructed using pET.AhgalE (harboring wild type galE) as a template (8). Oligonucleotides to generate mutants, Y149G, K153N and Y149G-K153N are shown in Table S1. The wild type and mutant proteins were expressed, purified and assayed for activity as described (8).

Modification of histidine, thiol and arginine residues of the 6xHis-rGalE

DEPC specifically modifies histidine(s) at pH 7.0 (4). Carbethoxylation using DEPC was carried out by incubating the enzyme in 20 mM potassium phosphate buffer, pH 7.0, with varying DEPC concentrations (prepared in ethanol). The rate of inactivation was determined by measuring the residual activity at different time intervals as described in Supplementary information (S.2). Reactivation of the modified 6xHis-rGalE was followed by 0.05 M hydroxylamine for 1 h. The percentage restored activity of the modified enzyme was determined in comparison of the native enzyme kept under identical conditions. To assess any adverse affect of DEPC on the coenzyme (NAD⁺), the modified and control samples were heated at 70°C for 15 min to dissociate NAD⁺ from the enzyme surface.

The activity of NAD⁺ in the supernatant was estimated by monitoring the reaction at 340 nm (16). Coenzyme fluorescence (excitation, 353 nm; emission, 435 nm), protein fluorescence (excitation, 290 nm; emission, 340 nm) and energy transfer spectrum (excitation, 290 nm; emission, 435 nm) of the control and modified enzyme (50 μg/ml) in 20 mM potassium phosphate buffer, pH 7.0, were recorded (Cary Eclipse Spectrofluorimeter; excitation and emission slit widths, 5 nm; scan speed, 50 nm/min). To ascertain the location of histidine residue, coenzyme fluorescence of the modified 6xHis-rGalE (1 mg/ml), incubated with 5′-UMP (1 mM) and sodium borohydride (20 mM NaBH₄) was measured.

For assessing the role of thiol residue in 6xHis-rGalE, varying concentrations of DTNB (20 mM sodium phosphate buffer; pH 8.0) at 25°C was added to the enzyme and residual activity was measured. Modification of the thiol residue was monitored at 412 nm. Reversal of DTNB-induced modification was performed by adding 50 mM DTT to the modified enzyme for 1 h. The enzyme’s residual activity and coenzyme fluorescence in the presence of another thiol modifying agent, NEM (10 mM) was also studied (6).

Arginine modification of the 6xHis-rGalE was carried out using 2,3 cyclobutanedione, in 50 mM sodium borate buffer, pH 8.8 at 28°C, followed by measuring the inactivation kinetics (5).

Biophysical characterization of the rGalE mutants

Far-UV circular dichroism (CD), fluorescence emission maxima and the denaturation transition of the 6xHis-rGalE and mutants were obtained as described in Supplementary information (S.3).

Thermal stability of the 6xHis-rGalE

Effect of glycerol on enzyme activity: The 6xHis-rGalE dia-lyzed against 20 mM Tris-HCl, pH 8.0, containing different concentrations of glycerol was heat inactivated at 45°C for 30 min, prior to activity assay (8). The 6xHis-rGalE without glycerol was included as control. The coenzyme and protein fluorescence was acquired as described above.

Tₘ measurement by spectroscopy and differential scanning calorimetry

The Tₘ of the 6xHis-rGalE (200 μg/ml) with and without glycerol was measured spectrophotometrically as described. Calorimetric measurements for same samples were performed on a VP-DSC (Microcal Inc., USA). The data were accumulated at a scan rate of 1°C/minute for 100 min. Tₘ was calculated by plotting Cp (kcal/mole) values on Y-axis versus temperature (°C) on the X-axis.

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REFERENCES