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Original Article

The ability of salts to stabilize proteins *in vivo* or intracellularly correlates with the Hofmeister series of ions

Fatmah M Hani*, Ashley E Cole*, Elliot Altman

*Department of Biology, Middle Tennessee State University, Murfreesboro 37132, TN, USA. *Equal contributors.*

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Abstract: Numerous studies have been conducted on the ability of salts to stabilize proteins *in vitro* using purified proteins demonstrating the fact that the ability of salts to stabilize proteins correlates with the Hofmeister series of ions. Using the well characterized bacterial aqueous cytosolic β-galactosidase and catechol 2,3-dioxygenase enzymes, we demonstrated that salts can stabilize proteins *in vivo* or intracellularly as well and that the ability of salts to stabilize these two proteins intracellularly also correlates with the Hofmeister series of ions. Na_2SO_4 and Na_2HPO_4 were very effective at stabilizing both proteins, followed by NaCl, NH_4Cl and $(NH_4)_2HPO_4$, while $NH_4CH_3CO_2$, $(NH_4)_2SO_4$ and $NaCH_3CO_2$ did not stabilize either of the proteins. We also investigated the ability of salts to rescue a collection of well characterized nonfunctional β-galactosidase and catechol 2,3-dioxygenase missense mutants that our laboratory has created. 73.33% of the β-galactosidase missense mutants could be rescued by salt, while only 33.33% of the catechol 2,3 dioxygenase missense mutants could be rescued by salt. This observation was explained by the differences in densities for the two proteins. Catechol 2,3 dioxygenase is almost twice as dense or compact as β-galactosidase and thus it is far easier for salts to penetrate and rescue inactive β-galactosidase proteins. 68.42% of the missense mutants that were rescuable by salt contained mutations that affected amino acids on the surface of the protein and is consistent with the likelihood that salt is able to rescue missense mutants that affect amino acids located on the surface of the protein much more readily than salt can rescue missense mutants that affect amino acids buried in the protein.

Keywords: Hofmeister ions, protein stability, salt suppression

Introduction

From the seminal studies of Hofmeister [1] regarding the ability of different salts to precipitate egg white protein, the lyotropic or Hofmesier series of ions was developed in which ions are ordered by their ability to precipitate proteins. For salts with the same cation, $SO_4^{2-} > HPO_4^{2-} > F^- > CH_3CO_2^- > Cl^- > Br > NO_3^- > I^- > ClO_4^- > SCN^-$ and for salts with the same anion, $(CH3)_4N^+ > Rb^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+}$. For general reviews see Jungwirth and Cremer [2] and Salis and Ninham [3]. Given the great significance of the Hofmeister series of ions, the 1888 Hofmeister article, which was written in German, has been translated in its entirety into English [4].

In a series of studies with collagen, gelatin and ribonuclease, Von Hippel and Wong [5-7] showed that the ability of the Hofmeister series of ions to precipitate proteins also correlated with their ability to stabilize proteins. Amongst the salts studied in these experiments, K_2HPO_4 and $(NH_4)_2SO_4$ had the greatest stabilizing effects, followed by KCl and NaCl. Subsequent studies by Nandi and Robinson [8, 9] using short peptides confirmed the fact that proteins were stabilized with respect to the Hofmeister series of ions. In these studies, Na_2SO_4 had the greatest stabilizing effect, followed by NaCl and NaBr. More recently Pelgram [10] also completed a thorough study on the ability of Hofmeister salts to stabilize the DNA binding domain (DBD) of *lac* repressor. In these studies, Na_2SO_4 and KF had the greatest stabilizing effects, followed by KCl and NaCl.

The ability of salts to stabilize proteins intracellularly has also been demonstrated in a number

of intracellular studies using nonfunctional missense mutants. Hawthorne and Friis [11] were the first to identify mutants whose functionality could be partially restored by the addition of salt as they demonstrated that 36 out of 231 *Saccharomyces cerevisiae* auxotrophic mutants could be rescued by the addition of KCl at 0.5 M or 1.0 M and proposed that salt correctable mutants were most likely to be missense mutants. Other researchers showed subsequently that this phenomenon occurred in bacteria as well [12-15]. Good and Pattee [12] isolated 15 temperature sensitive *Staphylococcus aureus* mutants that could be corrected by 1 M NaCl. Russell [13] demonstrated that 5 out of 14 temperature sensitive *Escherichia coli* mutants could be rescued by 1% (0.171 M) NaCl. Bilsky and Armstong [14] found that 32 out of 40 temperature sensitive *E. coli* mutants could be rescued by 0.5% (0.086 M) NaCl. Kohno and Roth [15] showed that 56 temperature sensitive and 47 cold sensitive *Salmonella enterica* serovar Typhimurium histidine auxotrophs could all by rescued by 0.2 M NaCl.

In this study, we tested the ability of Hofmeister salts to stabilize wild-type proteins intracellularly. The well characterized bacterial aqueous cytosolic β-galactosidase and catechol 2,3-dioxygenase enzymes were chosen for this analysis. The structures for both of these proteins has been determined [16, 17] and their activity can be quantified using robust easy to use colorimetric enzyme assays [18, 19]. We also examined the ability of salts to rescue a collection of inactive missense mutants that our laboratory has generated in these two proteins [20].

Materials and methods

Media and bacterial strains

Lysogeny broth (LB) [21] was used as the rich media to maintain the strains used in this study. While most studies that have been conducted in *E. coli* and *S. enterica* on the ability of salt to rescue nonfunctional missense mutants have used nutrient broth (NB), the basal salt concentration in nutrient broth is higher than desired. The concentration of sodium and chlorine in NB are 0.007 M and 0.003 M, respectively [22]. For this reason, we utilized yeast glucose broth (YGB), which consisted of 5.0% yeast extract supplemented with 0.2% glucose, as the rich medium in which all the strains were grown to determine the effects of the different salts that were tested. The concentration of sodium and chlorine in YGB are 0.003 M and 0.001 M, respectively [22] and thus the basal salt concentration in YGB is considerably lower than NB. Strains that were grown in YGB yielded saturated cultures with similar OD_{550} values to strains that were grown in NB or LB. To induce the expression of either the β-galactosidase enzyme coded by *lacZ* or the catechol 2,3-dioxygenase enzyme coded by *xylE*, isopropyl β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM using the *S. enterica* strains TT18519, *hisC10081*::MudF(*lac*+) and ALS1442, *proB*::*xylE*(*cat*), respectively [19]. Salt solutions to be tested were prepared at 1M concentrations, adjusted to pH 7 if necessary, and added to 2X YGB to achieve the final concentration desired in 1X YGB.

β-galactosidase and catechol 2,3-dioxygenase enzyme assays

β-galactosidase and catechol 2,3 dioxygenase assays assays were performed as described [18, 19] with two important modifications to eliminate the salt that is present in the buffers used in the assays. Z buffer was replaced with 10 mM Tris; pH 7 and both the ortho-nitrophenyl-β-galactoside (*ONPG*) and the catechol substrate solutions were prepared in 10 mM Tris; pH 7 instead of phosphate buffer.

Three dimensional analysis of the β-galactosidase and catechol 2,3-dioxygenase proteins

The PyMOL Molecular Graphics System (Schrödinger, LLC) was used to determine the volume of the β-galactosidase and catechol 2,3-dioxygenase proteins. The Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) was the source of the three dimensional crystal structure data. The 1DP0 file was used for the β-galactosidase crystal structure data [17] and the 1MPY file was used for the catechol 2,3-dioxygenase crystal structure data [16].

Results

Determining the ability of different salts to stabilize the β-galactosidase and catechol 2,3-dioxygenase enzymes intracellularly

We selected eight salts, $NH_4CH_3CO_2$ (ammonium acetate), NH_4Cl (ammonium chloride),

Table 1. Impact of different concentrations of Hofmeister salts on the activity of β-galactosidase and catechol 2,3-dioxygenase

Salts	0 M	0.05 M	0.1 M	0.15 M	0.2 M	0.25 M	0.3 M	0.35 M	0.4 M
β-galactosidase									
$NH_4CH_3CO_2$	152.11	33.53	25.08	19.59	9.39	ND	ND	ND	ND
NH_4Cl	152.11	179.73	174.20	150.20	140.81	141.21	140.26	139.84	136.25
$(NH_4)_2HPO_4$	152.11	171.10	105.94	97.84	84.16	81.42	32.19	30.82	20.84
$(NH_4)_2SO_4$	152.11	24.41	43.91	65.34	110.49	105.71	103.46	101.93	98.44
$NaCH_3CO_2$	152.11	88.42	80.72	95.50	110.18	83.64	80.11	76.62	74.15
NaCl	152.11	233.40	225.28	232.12	252.71	248.30	255.90	277.20	311.02
Na_2HPO_4	152.11	303.21	366.04	384.11	392.20	395.90	409.15	392.54	387.37
Na_2SO_4	152.11	323.43	390.31	473.60	604.90	659.46	770.16	777.96	667.50
Catechol 2,3-dioxygenase									
$NH_4CH_3CO_2$	344.79	32.21	25.39	21.63	22.61	ND	ND	ND	ND
NH_4Cl	344.79	368.45	413.99	464.59	464.05	442.13	399.63	428.02	483.88
$(NH_4)_2HPO_4$	344.79	658.53	643.23	635.43	613.40	484.03	301.61	247.72	117.15
$(NH_4)_2SO_4$	344.79	67.80	333.54	303.95	258.16	152.99	72.86	68.49	70.19
$NaCH_3CO_2$	344.79	34.86	21.40	17.99	18.88	18.33	10.38	2.69	3.17
NaCl	344.79	307.45	393.44	385.58	397.93	387.37	389.36	375.36	367.17
Na_2HPO_4	344.79	739.92	847.58	852.98	852.33	884.97	858.91	897.50	952.78
Na_2SO_4	344.79	322.28	415.87	442.38	551.09	881.66	922.25	977.02	142.65

β-galactosidase activities are reported in Miller Units and catechol 2,3-dioxygenase activities are reported in XylE Units. All assays were repeated in triplicate and the standard deviation was less than 10%. ND denotes that these values could not be determined. $NH_4CH_3CO_2$ completely inhibited cell growth at concentrations of 0.25 M or above.

$(NH_4)_2HPO_4$ (ammonium phosphate dibasic), $(NH_4)_2SO_4$ (ammonium sulfate), $NaCH_3CO_2$ (sodium acetate), NaCl (sodium chloride), Na_2HPO_4 (sodium phosphate dibasic) and Na_2SO_4 (sodium sulfate), for these studies based on three criteria. First, they contained both very strong and moderate anions and cations for stabilizing proteins according to the Hofmeister series of ions. Second, these were generally the best salts for stabilizing proteins based on previous studies [5-10]. Third, of the anions and cations that were selected, all of the possible combinations were tested.

The wild-type strains that produced β-galactosidase and catechol 2,3-dioxygenase were grown in YGB plus 1 mM IPTG that contained increasing salt in 0.05 M increments. After 16 hours of growth β-galactosidase or catechol 2,3-dioxygenase enzyme assays were performed and the results are shown in **Table 1**. Na_2SO_4 was the best salt for stabilizing β-galactosidase, followed by Na_2HPO_4, NaCl, NH_4Cl and $(NH_4)_2HPO_4$. $NH_4CH_3CO_2$, $(NH_4)_2SO_4$ and $NaCH_3CO_2$ did not stabilize β-galactosidase. Na_2SO_4 was the best salt for stabilizing catechol 2,3-dioxygenase, followed by Na_2HPO_4, $(NH_4)_2HPO_4$, NH_4Cl and NaCl. $NH_4CH_3CO_2$, $(NH_4)_2SO_4$ and $NaCH_3CO_2$ did not stabilize catechol 2,3-dioxygenase. Thus there was very good agreement in the ability of the various salts to stabilize both proteins. The molar concentrations at which the different salts provided their maximal stabilization varied widely and ranged from 0.05 M to 0.35 M for both enzymes.

Determining the ability of salt to suppress β-galactosidase and catechol 2,3-dioxygenase missense mutants

Numerous researchers have shown that nonfunctional missense mutants can be rescued by salt *in vivo*. We wanted to extend these studies by determining whether a collection of 42 unique well-characterized nonfunctional missense mutants, 15 in β-galactosidase and 27 in catechol 2,3-dioxygenase, could be rescued by salt. Because NaCl has been the salt of choice in the previous salt correctable missense mutant studies we used NaCl as well. Both 0.2 M and 0.3 M final concentrations of NaCl were tested to see if salt could rescue the missense mutants. **Table 2** shows the results

Table 2. Enzymatic activity of the salt correctible *lacZ* and *xylE* missense mutants in the presence of 0.2 M or 0.3 M NaCl

Mutant	0.0 M NaCl	0.2 M NaCl	0.2 M NaCl Fold Increase	0.3 M NaCl	0.3 M NaCl Fold Increase
β-galactosidase					
lacZ39	1.29	25.39	19.68	34.24	26.54
lacZ337	0.65	1.03	1.58	0.90	1.38
lacZ364	0.48	1.04	2.17	0.86	1.79
lacZ2234	0.70	0.89	1.27	0.91	1.30
lacZ2343	14.28	77.96	5.46	87.54	6.13
lacZ2381	0.78	2.37	3.04	2.67	3.42
lacZ2396	0.24	0.43	1.79	0.31	1.29
lacZ2449	1.26	14.27	11.33	12.41	9.85
lacZ2456	1.20	10.07	8.39	10.18	8.48
lacZ2530	0.79	2.42	3.06	2.61	3.30
lacZ2540	0.75	2.26	3.01	2.24	2.99
Catechol 2,3-dioxygenase					
xylE1568	7.46	10.40	1.39	14.17	1.90
xylE1569	20.59	25.69	1.25	30.58	1.49
xylE1583	8.34	11.24	1.35	11.82	1.42
xylE1585	8.17	17.67	2.16	15.00	1.84
xylE1591	14.16	20.34	1.44	26.75	1.89
xylE1683	6.76	16.82	2.49	11.77	1.74
xylE1698	7.42	10.41	1.40	15.56	2.10
xylE1702	9.60	14.14	1.47	21.09	2.20
xylE1710	6.18	10.92	1.77	11.61	1.88

β-galactosidase activities are reported in Miller Units and catechol 2,3-dioxygenase activities are reported in XylE Units. All assays were repeated in triplicate and the standard deviation was less than 10%.

of the enzyme assays for missense mutants that had at least a 25% or 1.25 fold increase in enzyme activity upon the addition of salt. Interestingly, NaCl had a negative effect on the missense mutants that were not rescuable by the addition of NaCl. 11 out of the 15, or 73.33% of the nonfunctional β-galactosidase missense mutants could be rescued by the addition of NaCl, but only 9 out of the 27, or 33.33% of the nonfunctional catechol 2,3-dioxygenase missense mutants could be rescued by the addition of NaCl. For the β-galactosidase missense mutants, the β-galactosidase activity was increased by an average of 5.53 fold with the addition of 0.2 M NaCl and 6.04 fold with the addition of 0.3 M NaCl. For the catechol 2,3-dioxygenase missense mutants, the catechol 2,3-dioxygenase activity was increased by an average of 1.64 fold with the addition of 0.2 M NaCl and 1.83 fold by the addition of 0.3 M NaCl. Thus the higher concentration of NaCl was slightly more effective at rescuing the nonfunctional missense mutants that could be rescued by salt. *lacZ39* was by far the most suppressible missense mutant as its β-galactosidase activity was increased 19.68 fold with the addition of 0.2 M NaCl and 26.54 fold with the addition of 0.3 M NaCl.

Using data from Cole *et al*. [20], **Tables 3** and **4** list the β-galactosidase and catechol 2,3-dioxygenase missense mutants, respectively, and gives the amino acid change, what secondary structure is affected by the missense mutant and what type of impact the mutation is expected to have on the secondary structure, whether the mutated amino acid is located on the surface of the protein, partially located on the surface of the protein or buried, the change in hydropathy caused by the mutation, and whether the missense mutant can be rescued by salt. Based on our knowledge of protein folding and structure, one would expect that missense mutants which affect amino acids located on the surface of the protein would be far more likely to be rescuable by salt than missense mutants which affect buried amino acids of the protein. 13 out of the 19, or 68.42% of the missense mutants that were rescuable by salt contained mutations on the surface of the proteins. Amongst the β-galactosidase missense mutants, where the large majority were rescuable by salt, the four missense mutants that could not be rescued by salt all contained mutations which affected amino acids that were buried in the protein.

Determining the ability of different salts to stabilize the β-galactosidase enzyme produced by the lacZ39 missense mutant

Because the *lacZ39* missense mutant was the most rescuable by NaCl, we tested the ability of the eight salts we had used previously with

Table 3. β-galactosidase missense mutants

lacZ mutant	Amino acid change[1]	Structural perturbation[2]	Mutation favorability[3]	Protein Location[4]	Change in hydropathy[5]	Salt Suppressible
39	1018 Leu to Gln	Beta Sheet	-	Surface (Partial)	+*	+
337	905 Glu to Lys	Coil	+	Surface (Partial)	+	+
361	900 Gly to Asp	Beta Sheet	-	Buried	+*	-
364	208 Gly to Asp	Beta Sheet	-	Buried	+*	+
2234	565 Gly to Asp	Beta Sheet	-	Buried	+*	+
2343	943 Arg to His	Beta Sheet	+	Surface	-*	+
2381	764 Gly to Asp	Coil	-	Buried	+*	+
2382	566 Gly to Asp	Beta Sheet	-	Buried	+*	-
2396	584 Gly to Asp	Coil	-	Surface	+*	+
2449	202 Asp to Asn	Coil	+	Surface (Partial)	-	+
2454	354 Gly to Asp	Beta Sheet	-	Buried	+*	-
2456	5 Thr to Met	Alpha Helix	+*	Surface	-*	+
2530	419 His to Tyr	Coil	-*	Surface	-	+
2540	4 Ile to Asn	Alpha Helix	-*	Surface	+*	+
2608	460 Gly to Arg	Coil	-*	Buried	+*	-

[1]The *lacZ* gene codes for the 1,024 amino acid β-galactosidase protein. The resulting amino acid changes are given based on the coded protein predicted by the DNA sequence and not the Protein Data Bank file. [2]The secondary structure affected by the mutation was determined using PyMol. Data from Jacobson *et al*. [23] had to be used to determine the secondary structures affected by *lac*Z2456 and *lac*Z2540, since these mutations were at the extreme amino terminus. [3]The likelihood of a mutation to affect the α-helical, β-sheet or random coil structure was determined using P_α, P_β or P_c values from the averaged propensity scale in Cole et al. [20]. (+) indicates a favorable change and (-) indicates an unfavorable change. An asterisk indicates a mutation that changes the original amino acid from or to one of the preferred amino acids that are found in α-helices, β-sheets or random coils. [4]The location of the mutated amino acids was determined using PyMOL. [5]The change in hydropathy was determined using the averaged hydrophobicity scale from Cole et al. [20]. (+) indicates a more hydrophilic change, (-) indicates a more hydrophobic change. An asterisk indicates a mutation that changes the hydropathy of the original amino acid significantly and results in a shift of at least 5 amino acids.

wild-type β-galactosidase and catechol 2,3-dioxygenase to see how they affected the inactive β-galactosidase enzyme produced by *lacZ39*. **Table 5** shows the result of this study. Na_2SO_4 was the best salt for stabilizing β-galactosidase from *lacZ39*, followed by NaCl, NH_4Cl, $(NH_4)_2SO_4$, $NaCH_3CO_2$, Na_2HPO_4 and $(NH_4)_2HPO_4$. Only $NH_4CH_3CO_2$ did not stabilize the β-galactosidase from *lacZ39*. The results clearly demonstrate that the salts affect wild-type β-galactosidase enzyme differently than an inactive mutant β-galactosidase enzyme. While Na_2SO_4 was the best salt at stabilizing both wild-type β-galactosidase and the β-galactosidase produced from *lacZ39*, NaCl was almost as effective at stabilizing the β-galactosidase produced from *lacZ39*. Neither $NH_4CH_3CO_2$, $(NH_4)_2SO_4$ or $NaCH_3CO_2$ could stabilize wild-type β-galactosidase, but only $NH_4CH_3CO_2$ was ineffective at stabilizing the β-galactosidase produced from *lacZ39*.

Discussion

In this study, we have examined whether the best salts known to stabilize purified proteins *in vitro* based on previous studies [5-10] could also perform *in vivo* or intracellularly using the two very well characterized β-galactosidase and catechol 2,3 dioxygenase enzymes. To ensure that all the possible combinations of anions and cations were tested based on the salts that have proven the most effective at stabilizing proteins in *in vitro* studies, we tested the following eight salts, $NH_4CH_3CO_2$ (ammonium acetate), NH_4Cl (ammonium chloride), $(NH_4)_2HPO_4$ (ammonium phosphate dibasic), $(NH_4)_2SO_4$ (ammonium sulfate), $NaCH_3CO_2$ (sodium acetate), NaCl (sodium chloride), Na_2HPO_4 (sodium phosphate dibasic) and Na_2SO_4 (sodium sulfate). Generally, Na_2SO_4 and Na_2HPO_4 were very effective at stabilizing both proteins, followed by NaCl, NH_4Cl and $(NH_4)_2HPO_4$, while $NH_4CH_3CO_2$, $(NH_4)_2SO_4$ and $NaCH_3CO_2$ did not stabilize either of the proteins. However, **Table 1** clearly shows some distinct differences in the ability of the different salts to stabilize the two proteins. Na_2SO_4 and Na_2HPO_4 are equally effective at stabilizing catechol 2,3 dioxygenase, but Na_2SO_4 is much more effective at stabilizing β-galactosidase than Na_2HPO_4. NaCl is

Table 4. Catechol 2,3-dioxygenase missense mutants

xylE mutant	Amino acid change[1]	Structural perturbation[2]	Mutation favorability[3]	Protein Location[4]	Change in hydropathy[5]	Salt Suppressible
1568	106 Arg to Cys	Beta Sheet	+*	Surface (Partial)	-*	+
1569	270 Gly to Gln	Coil	-*	Surface (Partial)	+*	+
1571	127 Gly to Gln	Coil	-*	Surface (Partial)	+*	-
1574	233 Ser to Phe	Alpha Helix	+	Surface	-*	-
1577	21 Ala to Val	Alpha Helix	-*	Buried	-*	-
1581	30 Gly to Ser	Coil	-	Surface (Partial)	+	-
1582	64 Gly to Asp	Beta Sheet	-	Surface	+*	-
1583	158 Gly to Asp	Beta Sheet	-	Buried	+*	+
1584	243 Pro to Ser	Coil	-	Surface (Partial)	+	-
1585	9 Gly to Ser	Coil	-	Buried	+	+
1587	178 Gln to Lys	Beta Sheet	+	Surface	+	-
1588	247 Gly to Asp	Coil	-	Surface (Partial)	+*	-
1589	282 Trp to Ser	Beta Sheet	-*	Surface (Partial)	+*	-
1591	8 Pro to Ser	Beta Sheet	+	Buried	+	+
1592	193 Ser to Asn	Beta Sheet	-	Buried	+	-
1593	245 Arg to Cys	Coil	+	Surface (Partial)	-*	-
1665	242 Gly to Asp	Coil	-	Surface (Partial)	+*	-
1666	251 Gly to Asp	Coil	-	Surface (Partial)	+*	-
1670	202 Ala to Thr	Beta Sheet	+*	Buried	+	-
1677	213 His to Tyr	Beta Sheet	+*	Buried	-	-
1680	216 Ser to Phe	Beta Sheet	+*	Buried	-*	-
1681	200 Asp to Asn	Coil	+	Buried	-	-
1683	115 His to Tyr	Coil	-*	Surface (Partial)	-	+
1684	137 Glu to Lys	Coil	+	Surface	+	-
1698	152 Asp to Asn	Coil	+	Buried	-	+
1702	24 His to Tyr	Alpha Helix	+	Surface (Partial)	-	+
1710	290 Ala to Thr	Alpha Helix	-*	Surface (Partial)	+	+

[1]The *xylE* gene codes for the 307 amino acid catechol 2,3-dioxygenase protein. The resulting amino acid changes are given based on the coded protein predicted by the DNA sequence and not the Protein Data Bank file. [2]The secondary structure affected by the mutation was determined using PyMol. [3]The likelihood of a mutation to affect the α-helical, β-sheet or random coil structure was determined using P_α, P_β or P_c values from the averaged propensity scale in Cole *et al*. [20]. (+) indicates a favorable change and (-) indicates an unfavorable change. An asterisk indicates a mutation that changes the original amino acid from or to one of the preferred amino acids that are found in α-helices, β-sheets or random coils. [4]The location of the mutated amino acids was determined using PyMOL. [5]The change in hydropathy was determined using the averaged hydrophobicity scale in Cole *et al*. [20]. (+) indicates a more hydrophilic change, (-) indicates a more hydrophobic change. An asterisk indicates a mutation that changes the hydropathy of the original amino acid significantly and results in a shift of at least 5 amino acids.

much better at stabilizing β-galactosidase than NH_4Cl and $(NH_4)_2HPO_4$, while $(NH_4)_2HPO_4$ is much better at stabilizing catechol 2,3 dioxygenase than NH_4Cl or NaCl.

Numerous researchers have shown that salt can rescue inactive proteins intracellularly by testing the ability of salt to suppress nonfunctional missense mutants [11-15]. We have expanded these studies by testing the ability of salt to rescue a set of 42 well characterized unique nonfuctional missense mutants, 15 in β-galactosidase and 27 in catechol 2,3 dioxygenase. A total of 19 out of the 42, or 45.24% of the missense mutants were rescuable by salt (11 out of 15, or 73.33% for β-galactosidase and 9 out of 27, or 33.33% for catechol 2,3 dioxygenase). Thus clearly it was a lot easier to suppress nonfunctional missense mutants in β-galactosidase than it was to suppress nonfunctional missense mutants in catechol 2,3 dioxygenase. An examination of the structure of the two proteins provides an answer.

Both the β-galactosidase and catechol 2,3 dioxygenase proteins are tetrameric. The β-gal-

Table 5. Impact of different concentrations of Hofmeister salts on the activity of β-galactosidase for the *lacZ*39 missense mutant

Salts	0 M	0.05 M	0.1 M	0.15 M	0.2 M	0.25 M	0.3 M	0.35 M	0.4 M
$NH_4CH_3CO_2$	1.29	.096	1.05	0.84	0.80	ND	ND	ND	ND
NH_4Cl	1.29	3.11	8.97	10.08	13.65	16.88	19.94	21.44	18.47
$(NH_4)_2HPO_4$	1.29	2.28	1.91	1.38	1.14	1.08	0.60	0.52	0.43
$(NH_4)_2SO_4$	1.29	3.74	12.07	14.42	17.02	2.26	1.91	1.74	1.01
$NaCH_3CO_2$	1.29	3.17	2.84	3.40	4.46	6.36	8.23	8.41	7.66
NaCl	1.29	7.06	10.34	16.06	25.39	31.38	34.24	35.31	40.25
Na_2HPO_4	1.29	3.83	4.49	4.55	4.17	3.39	2.90	2.58	2.52
Na_2SO_4	1.29	11.17	22.11	36.39	57.26	48.67	45.18	39.15	34.64

β-galactosidase activities are reported in Miller Units. All assays were repeated in triplicate and the standard deviation was less than 10%. ND denotes that these values could not be determined. $NH_4CH_3CO_2$ completely inhibited cell growth at concentrations of 0.25 M or above.

actosidase monomer is 1,024 amino acids in size and the tetramer's molecular weight is 465,912 g/mol with width, height and depth measurements of 174.10 Å, 136.00 Å and 86.75 Å, respectively. The catechol 2,3 dioxygenase monomer is 307 amino acids in size and the tetramer's molecular weight is 140,616 g/mol with width, height and depth measurements of 94.95 Å, 65.70 and 51.60 Å, respectively. Thus the density of β-galactosidase is 0.377 g/cm^3, while the density of catechol 2,3 dioxygenase is 0.725 g/cm^3. Since β-galactosidase is a lot less dense or compacted than catechol 2,3 dioxygenase, it should be a lot easier for salts to penetrate and effect β-galactosidase than catechol 2,3 dioxygenase. Additionally, β-galactosidase has a unique structure that consists of a continuous system of channels running along the surface and within the tetramer. These channels appear to be accessible to bulk solvent and vary in width from 5-20 Å [17].

Which β-galactosidase and catechol 2,3 dioxygenase missense mutants were rescuable by salt can also be explained by considering the structures of the proteins. 13 out of the 19, or 68.42% of the missense mutants that were suppressible contained amino acid changes that were located on the surface of the proteins and thus would be expected to be more rescuable by salt. For β-galactosidase in which 11 out of 15, or 73.33% of the missense mutants were suppressible by salt, the only missense mutants that were not suppressible affected amino acids that were buried in the protein.

Several studies have demonstrated the propensity of Hofmeister salts to stabilize proteins via hydrophobic interactions [24-26] and that small cations and large anions facilitate this effect [27]. According to these findings NaCl, which has a smaller cation with an ionic radius of 0.102 nM and a larger anion with an ionic radius of 0.181 nM, would be expected to rescue inactive missense mutants where the mutated amino acid had a hydrophobic shift to a greater degree than missense mutants where the mutated amino acid had a hydrophilic shift. Of the 42 β-galactosidase and catechol 2,3 dioxygenase missense mutants that were characterized in this study, 14 of the mutants had an amino acid change that resulted in a hydrophobic shift and 28 of the missense mutants had an amino acid change that resulted in a hydrophilic shift. A total of 8 out of the 14, or 57.14% of the mutants that resulted in a hydrophobic shift were rescuable by NaCl, while only 12 out of 28, or 42.86% of the mutants that resulted in a hydrophilic shift were rescuable by NaCl. Thus there was a slight preference for NaCl to rescue mutants that resulted in a hydrophobic shift than to rescue mutants that resulted in a hydrophilic shift.

We further characterized the ability of the different salts to correct the *lacZ*39 missense mutant that was highly suppressible by salt. Na_2SO_4 was the most effective salt for stabilizing β-galactosidase from lacZ39, followed by NaCl, NH_4Cl, $(NH_4)_2SO_4$, $NaCH_3CO_2$, Na_2HPO_4 and $(NH_4)_2HPO_4$. Only $NH_4CH_3CO_2$ could not stabilize the β-galactosidase from lacZ39. Interestingly, unlike wild-type β-galactosidase where NaCl was not nearly as effective as Na_2SO_4, NaCl was almost as effective as Na_2SO_4 at stabilizing the β-galactosidase produced by lacZ39. This finding is consistent with

NaCl being the choice of most researchers that have tested the ability of salts to rescue non-functional missense mutants.

Historically, most researchers have used NaCl to form the salt gradients in the anionic exchange or hydroxyapatite columns used in fast protein liquid chromatography (FPLC) to purify proteins and the resulting proteins are stored in buffers containing NaCl. The results of our intracellular studies and the numerous *in vitro* studies by other researchers that have investigated the ability of salts to stabilize proteins, suggest that Na_2SO_4 and Na_2HPO_4 would be better choices for stabilizing proteins.

Disclosure of conflict of interest

None.

Address correspondence to: Elliot Altman, Department of Biology, Middle Tennessee State University, Murfreesboro 37132, TN, USA. Tel: 615-710-8616; E-mail: ealtman@mtsu.edu

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