The Effect of Buffers on Protein Conformational Stability

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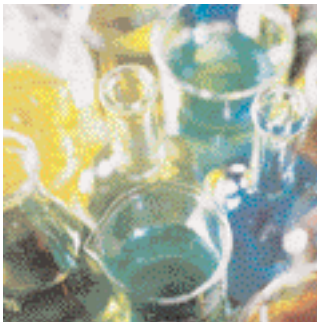

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The extent to which a particular protein may be stabilized or destabilized by a buffer depends on many factors, thereby making the selection of a buffer for formulating a specific protein a formidable challenge. The authors describe qualitative and semiqualitative correlations to help in the selection of a buffer for a particular protein and formulation.

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Buffers used to formulate proteins should not serve as substrates or inhibitors. They should exhibit little or no change in pH with temperature, show insignificant penetration through biological membranes, and have maximum buffer capacity at a pH where the protein exhibits optimal stability. In conformity with the proposition that "Nature designs the optimum molecules," buffers should mimic the antidenaturant properties of nature exhibited by osmolytes (1–5) that are independent of the evolutionary history of the proteins (6, 7). Such properties may include preferential exclusion from the protein domain (8–11) and stabilization without changing the denaturation Gibbs energy (ΔG_d) (12).

Conformational instability refers not only to unfolding, aggregation, or denaturation but also to subtle changes in localized protein domains and the alteration of enzyme catalytic properties (13) that may result from buffer-component binding, proton transfer, and metal or substrate binding effects directly or indirectly mediated by buffers or by buffers themselves acting as pseudosubstrates.

Salts can affect protein conformation to the extent that the anions or cations of the salt could be potential buffer components. When the salt concentration is much larger than that of the buffer, the salt becomes the effective buffer in the reaction.

The mechanisms or combinations thereof by which buffers may cause protein stabilization (or destabilization) are complex and not well understood. The problem is compounded by the inability to definitively differentiate between various protein stabilization mechanisms, the small free energies of stabilization of globular proteins (14–16), and a paucity of review manuscripts on this subject in the literature. The authors address some of these issues as they relate to buffers used in the formulation of proteins. The effect of buffers that may be used in the extraction, purification, dialysis, refolding, or assay of proteins on protein conformation is not discussed.

Buffer effects on freeze drying

Change in pH as a result of buffer salt crystallization. When inorganic salts are used as buffers, the freezing point of the monoionized species (salt) can be different from that of the nonionized (i.e., free acid or base) species and from its higher ionized species. This difference leads to the freezing of one form before

List of abbreviations

ACES: *N*-2-acetamido-2-aminoethane sulfonic acid
2,3-BPG: 2,3-bis phosphoglycerate
CHES: 1-[*N*-cyclohecyamino]-ethane sulfonic acid
CLARP: caspase-like-apoptosis-regulatory-protein
DIPSO: 3-[*N*,*N*-bis(hydroxyethyl)amino]-2-hydroxypropane sulfonic acid
G-CSF: granulocyte colony stimulating factor
Good's buffers: zwitterionic buffers containing aminoalkyl sulfonate (e.g., DIPSO, MES, HEPPSO, HEPES)
HEPES: 4-(2-hydroxyethyl)piperazine-*N*′-2-ethane sulfonic acid
HEPPSO: [*N*-(2-hydroxyethyl)piperazine-*N*′-2-hydroxypropane sulfonic acid
KCl: potassium chloride
NaCl: sodium chloride
NADPH: nicotinamide adenine dinucleotide phosphate
Na_2HPO_4: disodium phosphate
$(NH_4)_2SO_4$: ammonium sulfate
NTB: nitrothiobenzoate
MES: 2-(*N*-morpholino)ethane sulfonic acid
MOPS: 3-(*N*-morpholino-2-hydroxypropane sulfonic acid
PIPES: [piperazine-*N*,*N*′-bis(ethane sulfonic acid)]
POPSO: piperazine-*N*,*N*′-bis(2-hydeoxypropane sulfonic acid)
PBS: phosphate buffered salineTAPS: *N*-tris[hydroxymethyl]methyl-3-aminopropane sulfonic acid
TEA: triethylamine
TES: 1% sodium dodecyl sulfate + 5mM EDTA + 10 mM TRIS-HCL
TRIS-HCL: Tris-(hydroxymethyl)aminomethane hydrochloride

the other during the freezing phase of lyophilization (17). Such a phenomenon has been linked to drastic changes in pH of the liquid medium during freezing, which can lead to the denaturation of the protein being lyophilized (18, 19). If an amphoteric molecule were to function as a buffer containing both acidic and basic groups on one molecule, one would expect negligible pH shifts to occur during the crystallization of this zwitterionic molecule (20). Such is indeed the case for various organic buffers broadly categorized as aminoalkylsulfonate zwitterions (21). Good et. al. prepared and disclosed such buffers in their classic publication (22).

Researchers have shown that replacing the Na^+ cation with the K^+ cation in a phosphate buffer could significantly decrease the pH shift during the freezing stage of lyophilization (23). A potassium phosphate buffer at pH 7.2 exhibited a eutectic point at a temperature greater than −10 °C. However, the sodium cation counterpart showed a eutectic point at a temperature below −20 °C. Monoclonal antibodies against HBV and L-selectin, humanized IgG, as well as monomeric and tetrameric β-galactosidase exhibited less aggregation when subjected to freeze–thaw cycles with a potassium phosphate buffer than with a sodium phosphate buffer (24). Similarly, the propensity of recombinant hemoglobin to denature as a result of phase separation from a polyethylene glycol–dextran matrix was reduced when NaCl was replaced with KCl in the formulation buffer. In this case, the sodium phosphate buffer did not exhibit a pH shift during freezing owing to inhibition of crystallization of disodium phosphate by the polymer (25). However, replacing NaCl with KCl did decrease the phase separation caused by annealing at −7 °C because of the propensity for KCl, but not NaCl, to form a stable glass at this temperature (26). Also, the specific surface area of freeze-dried bovine IgG from solutions containing NaCl was found to be significantly higher than those containing KCl (27). Annealing also increases the surface accumulation of proteins at the ice–liquid interface so that the formation of a stable glass at the annealing temperature is especially important to minimize denaturation caused by such a mechanism (28).

The rate of aggregation of recombinant human interleukin-1 receptor antagonist (rhIl-1ra) was greater in mannitol–phosphate formulations than in glycine–phosphate formulations, possibly owing to the inhibition of the selective crystallization of the dibasic salt by glycine during freezing, thereby preventing large localized pH changes in the frozen matrix (29).

The effect of various buffer solutions on freezing damage to rabbit-muscle-derived lactate dehydrogenase, type II (LDH, isoionic point [pI] = 4.6) was examined with sodium phosphate, TRIS-HCl, HEPES, and citrate buffers (50 mM, pH 7.0) and pH 7.4 (30). The activity recovery was directly proportional to enzyme concentration and was the lowest in the sodium phosphate buffer (31). The activity increased in the following order: citrate < Tris ≈ potassium phosphate < HEPES. The low activity recovery in the sodium phosphate buffer was attributed to its significant pH shift on freezing (32). The study revealed no clear pattern relating recovery of activity after freezing to the freezing rate because an intermediate freezing rate gave the highest recovery of activity (31). The researchers hypothesized that the slowest freezing method actually resulted in a greater degree of supercooling and better thermal equilibration throughout the volume of liquid such that, when ice crystals nucleated, the freezing rate actually was faster than designed. Therefore, the study illustrates the need to control the extent of supercooling by seeding the cooling liquid when comparing the effects of buffers or lyoprotectants on the stability of freeze-dried proteins.

In another study, the decreased solid-state stability of lyophillized recombinant human interferon γ (pI = 10.3) in sodium succinate buffer as compared with sodium glycolate buffer was attributed to a pH shift occuring in the former (19). In this case, however, the decrease in pH on freezing was attributed to crystallization of the monosodium form of succinic acid. In addition, it was not immediately clear why a similar crystallization effect would not be observed with the use of sodium salt of the glycolic acid. In any event, the Na^+ cation can be replaced with the K^+ cation in inorganic buffers as a first approximation to potentially increase the stability of proteins during the freezing stage of lyophillization.

Influence on specific surface areas of lyophillized cakes. An aggregation mechanism involving partial denaturation at the ice–freeze concentrate interface has also been linked to an increase in protein degradation (27). Denaturation induced by this mechanism can be reduced by incorporating surface active agents in the formulation (33). Studies have shown the copper complexing ability of several zwitterionic N-substituted aminosulfonic acid buffers to correlate with surface activity at pH 8.0 as measured by alternating current polarography (34).

Increasing surface activity was correlated to the number of hydroxyl groups; thus, the following order: DIPSO (three hydroxyl groups) > HEPPSO, POPSO (two) > PIPES (no hydroxy groups), showing no surface activity.

In another study, the increase of Cu^{+2} toxicity observed in the marine dinoflagellate *A. carterae* in the presence of more than 10 mM HEPES at pH 8.0 was attributed to the surfactant activity of this buffer, as measured using an array of various electrochemical techniques (35). Such buffers exhibiting some degree of surface activity could be used to potentially inhibit freeze-induced damage to proteins that involve the partial unfolding of proteins after adsorption to the ice surface.

Influence on thiol–disulfide interchange. The aggregation of lyophilized natriuretic peptide (ANP, pI > 10) was significantly reduced when the concentration of acetic acid buffer at pH 4.0 was increased from 5 to 15 mM before lyophilization (36). The mechanism of aggregation was attributed to alkali induced β-elimination from the disulfide linkage to form a free thiolate ion. The thiolate anion subsequently underwent thiol–disulfide interchange with ANP to form the disulfide-linked multimers. However, it was not apparent why a phase transition of ostensibly incompletely crystallized mannitol after lyophilization from a glass to a crystal upon storage would trigger an increase of local pH in the lyophilized product (that was attributed to the generation of thiolate ions).

Influence on excipient properties of crystallinity and glass transition. β-galactosidase was lyophilized in a range of sodium phosphate buffer concentrations (10–200 mM, pH 7.4) containing varying amounts of mannitol (0–500 mM). A larger mannitol concentration without buffer caused aggregation presumably as a result of the complete crystallization of mannitol. The residual activity was preserved at buffer–mannitol concentrations at which the buffer presumably prevented the crystallization of mannitol (37).

The glass-transition temperature of lyophilized rhIl-1ra containing 1% sucrose, 4% mannitol, and 2% glycine decreased from 46 to 26 °C when the buffering agent sodium citrate was replaced with sodium phosphate (38). This result was consistent with the observation that the lyophilized product was more stable in citrate than in phosphate buffer containing these excipients.

Chaotrope–Kosmotrope effects

Chaotropic anions are water-structure breakers and destabilize proteins. Kosmotropic anions are polar water-structure makers and generally stabilize proteins (39–41). A study involving aqueous column chromatography on a size-exclusion cross-linked dextran gel showed that a chaotrope interacts with the first layer of immediately adjacent molecules somewhat less strongly than would bulk water in its place and that a polar kosmotrope interacts more strongly (42). The ability of anions to make or break water structure closely parallels the Hofmeister series (43). A continual decay in activity of an immobilized fusion protein (organophosphorus hydrolase of pI = 8.3 and green fluorescent protein) was observed in reaction mixtures containing 1-[*N*-cyclohexylamino]-ethane sulfonic acid (CHES) at pH 6.9. This decay in activity was fully restored, along with fluorescence, upon washing with PBS buffer. The researchers concluded that the sulfonate was more chaotropic than the phosphate anion (44).

The solution and thermal stability of the tetrameric enzyme, phosphoenolpyruvate carboxylase (PEPC, pI = 6.0) was determined at pH 6.2 in MES buffer in the presence of various salts by temperature-accelerated enzyme activation and by size exclusion chromatography (45). Results showed that kosmotropic anions (HPO_4^{-2}, citrate^{-3}, SO_4^{-2}, F^-, OAc^-) and glutamate stabilized the enzyme most effectively and that Cl^- and Br^- were destabilizing. The effect of cations ranged from relatively inert (e.g., Na^+ and K^+) to destabilizing (e.g., NH_4^+, Li^+, $(CH_3)_4N^+$). The observed stabilization by specific salts was interpreted in terms of the positive surface-tension increment and the water-structuring effects conferred on the solution by these agents. The destabilization by some salts was associated with the dissociation of the tetrameric enzyme into its dimeric and monomeric forms.

Effect of buffer heat of ionization

When protein conformation is protonation dependent (i.e., the enthalpy of denaturation or the association constant—for binding between substrate and ligand—varies with pH), the observed denaturation or binding enthalpy often varies with the kind of buffer used in the study. This variation exists because the experimentally measured enthalpy (ΔH_{obs}) at a given pH is determined by two values: the ionization enthalpy of the particular buffer used (ΔH_{ion}) and the enthalpy of the denaturation or binding process corrected for buffer effects (ΔH_b). These enthalpies are related to ΔH_{obs} by

$$\Delta H_{obs} = \Delta H_b + (n)\Delta H_{ion}$$

in which n is the number of protons released (positive sign) or taken up (negative sign) by the buffer during denaturation or binding.

A graph of ΔH_{ion} against ΔH_{obs} at various pH values (46) (see Figure 1) can be used to obtain the "true" enthalpy change on denaturation or binding, to "deconvolute" the enthalpic and entropic components of reactions involving a change in protein conformation, and to estimate the pKa values of the ionizable group(s) in the protein involved in the reaction.

A similar study was undertaken to investigate the possibility that the uptake or release of protons was responsible for the anomalous heat-capacity change obtained during complexation of dihydrolipoyl acetyltransferase to dihydrolipoyl dehydrogenase in the multienzyme complex of *Bacillus stearothermophilus* (47). The effect of the buffer heat of ionization was similarly studied for the acylation of α-chymotrypsin (48), the binding of NADPH to α-ketoglutarate (49), and the hexokinase-catalyzed phosphorylation of sugars by ATP (50).

Studies on the helix-forming thermodynamic propensity scales of various amino acid residues indicate that an amino acid residue located at a solvent-exposed position of an α helix differently affects the stability of the protein (51–53). Such stabilization in proteins is very similar to that found in short helical peptides of the same amino acid sequence, thereby indicating the fundamental character of the observed thermodynamic propensity

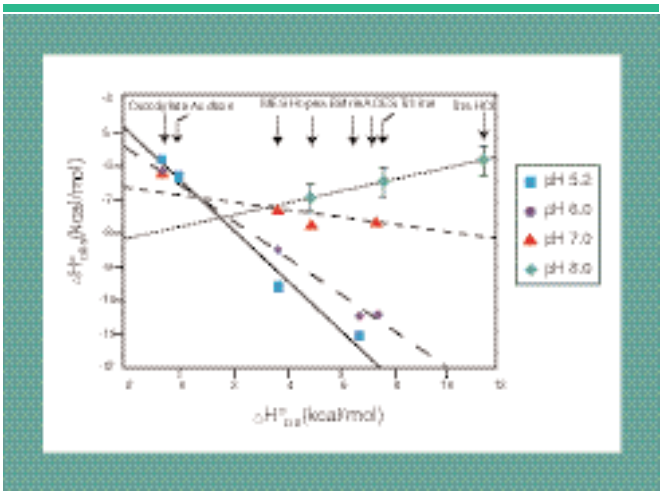

Figure 1: Dependence of protein unfolding on the heat of ionization of buffer. Figure shows enthalpies of binding for the Src SH2 domain binding to the hmT peptide at various pH values as a function of ΔH_{ion} of the buffer (adapted from Reference 46).

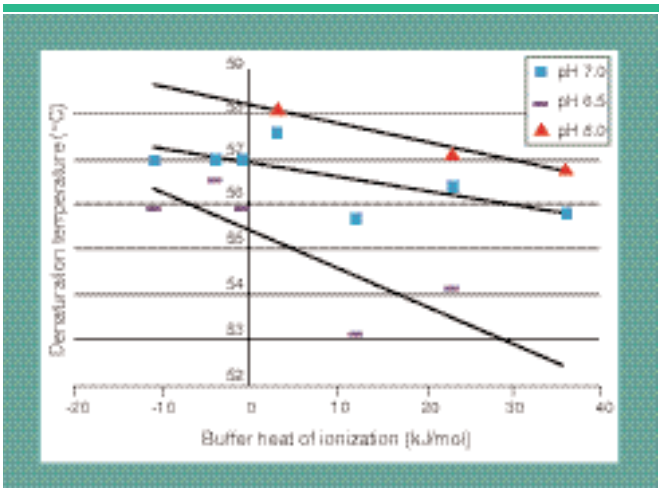

Figure 2: CspA unfolding in various buffers (adapted from Reference 56).

(54, 55). Where the unfolding of such proteins appears to be linked to the protonation of a solvent-exposed amino acid residue—such as that of the major cold-shock protein of *Escherichia coli* CspA—studies have shown that the magnitude of the denaturation temperature is inversely correlated with the buffer's heat of ionization (56) (see Figure 2). The figure shows the following buffers and their heats of ionization (kJ/mol): cacodylate (−4), phosphate (−1), HEPES (+3), citrate (−11), PIPES (+12), MOPS (+23), and imidazole (+36).

The antioxidant effect of buffers

Some Good's buffers are efficient scavengers of hydroxyl radicals with rate constants of ~10^9 /M s (57). Tris, tricine, and HEPES (in that order) were shown to inhibit the loss of a competitive solute, thymine, in radiolyzed water. HEPES and Tris but not phosphate inhibited the rate of auto-oxidation of hemoglobins A (pI = 6.9) and S (58). The mechanism was not specifically attributed to free radical scavenging but rather by the binding of the phosphate anion to the 2,3-BPG binding site at pH 7.0. This binding favored a shift to the deoxy state that was linked to more rapid methemoglobin formation. In contrast, HEPES and Tris, being positively charged at pH 7.0, were not bound as readily as phosphate to the 2,3-BPG electropositive region in the hemoglobin molecule. HEPES and MOPS also accelerated the decomposition rate of the oxidant, peroxynitrite ($ONOO^-$) (59). The ability of $ONOO^-$ to stimulate current good manufacturing practice (CGMP) formation in cultured endothelial cells in the presence of HEPES and MOPS but not phosphate was attributed to the oxidant's reaction with the buffers to release NO in a Cu(I) catalyzed reaction (60). In contrast, the binding of phosphate or phosphorylated compounds to acidic fibroblast growth factor (aFGF) significantly reduced the copper catalyzed oxidation of its free thiol groups, thereby reducing aggregation (61).

The rate of Fe(II) auto-oxidation was substantially larger in phosphate and bicarbonate than in HEPES, MOPS, Tris, or MES buffers (50 mM, pH 6.5–7.0). Furthermore, the rate of Fe(II) auto-oxidation of Fe(II) chelates with oxygen ligands was higher than the auto-oxidation rate of Fe(II) chelates with nitrogen ligands (62). Results indicated that phosphate buffer could chelate with Fe(II), thereby promoting its oxidation even in the absence of free hydroxyl radicals (63). In addition, another study was conducted to compare the hydroxyl radical quenching ability of phosphate, carbonate, and bicarbonate buffers. Results showed that phosphate buffer quenched hydroxyl radicals less efficiently than did carbonate or bicarbonate buffers (64).

The ability of buffers to scavange free radicals assumes increased importance with the emergence of depot protein formulations administered by intramuscular or subcutaneous injection in conjunction with the absence of glycosylation in recombinantly produced proteins. Nonglycosylated proteins are more prone to denaturation by free radical attack than O-linked glycosylated proteins (65–67). However, studies have not recognized that other factors such as protection against free-radical–mediated denaturation and/or a decrease in the amide proton exchange rate may have been partly responsible for the sustained activity of recombinant bovine granulocyte colony stimulating factor (rbG-CSF, pI = 6.6), when formulated in organic buffers rather than in acetate buffer (68, 69).

Buffer effect on thiol–disulfide interchange reactions

Proteins administered through a controlled-release system such as polymeric matrices containing powdered proteins are exposed to high water activity (70). The moisture-induced aggregation of several proteins is caused by intermolecular *s–s* bond formation via the thiol–disulfide interchange reaction (31). The aggregation of bovine serum albumin caused by such reaction is substantially reduced when the initiating buffer (5 mM phosphate, 150 mM NaCl, pH = 7.3) contained 1 M sodium phosphate (71). The addition of 4 M NaCl did not cause the same level of inhibition as the sodium phosphate. Because the thiolate anion (rather than the thiol) is the reactive species in the thiol–disulfide interchange, it is possible that the phosphate anion prevents the nucleophilic attack of the thiolate anion on the disulfide linkage (72, 73). This effect may be partly a result of charge repulsion because in at least another case, in-

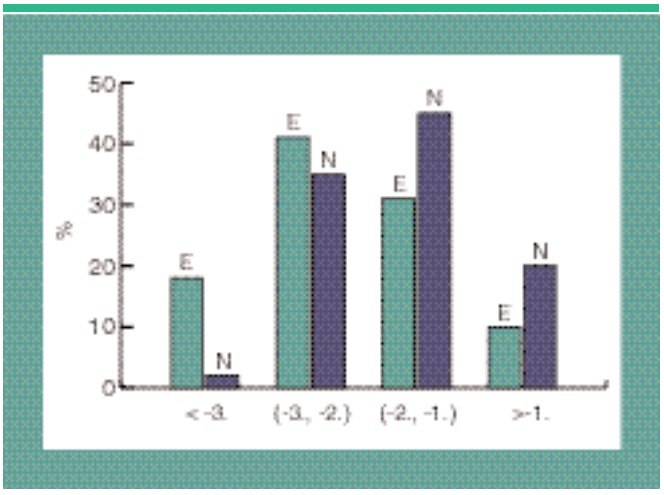

Figure 3: Optimization of charge–charge interactions by Monte Carlo analysis (adapted from Reference 74).

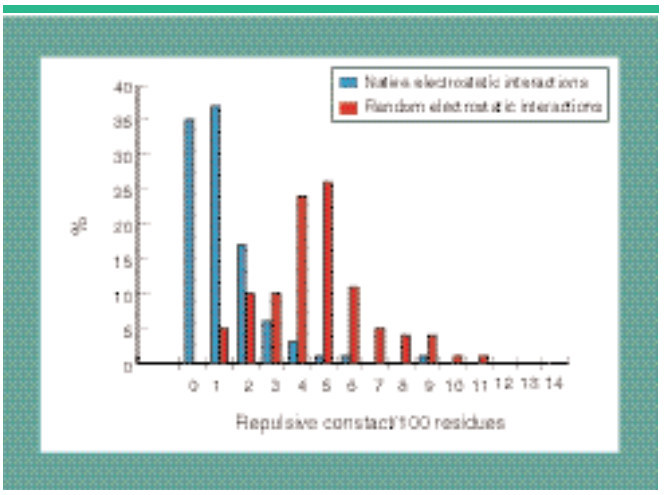

Figure 4: Electrostatic interactions of native protein (adapted from Reference 74).

creasing the concentration of buffer (acetate) also seemed to decrease the extent of thiol–disulfide interchange (36).

Effect of buffer–salt concentration

An excellent review showed that charge–charge interactions were better optimized in the enzymes (E) than in proteins without enzymatic functions (N), relative to randomly distributed charge constellations obtained by the Monte Carlo technique (74) (see Figure 3). Proteins belonging to the mixed αβ type were electrostatically better optimized than pure α-helical or β-strand structures. Proteins stabilized by disulfide bonds showed a lower degree of electrostatic optimization. Finally, the electrostatic interactions in a native protein were effectively optimized by rejection of the conformers that lead to repulsive charge–charge interactions (see Figure 4). The implication of this computational analysis is that salt or buffer-mediated electrostatic or binding effects are likely to be more pronounced in enzymes rather than in proteins; in higher evolutionary folding classes that use the α/β or the α + β folds rather than in pure α or pure β folds; and in proteins that have relatively fewer disulfide bonds in their primary structure, all other factors being equal (75).

The larger the difference between the pI and the pH of interest, the greater the net charge on the protein. This implies that the ability of ionic compounds to cause either stabilization or destabilization of the protein by binding to specific residues (not kosmotropic or chaotropic effects) should increase as the difference between pI and pH becomes greater (76). This effect is even greater for proteins in which the ionic contributions substantially affect protein stability (see Figure 3). Classical protein electrostatics dictates that the electrostatic contributions to stability should be maximal at the pI and hence the salt dependence on stability at a given pH also should be determined by the distance to the isoionic point, pI. The difference in estimates of the stability of a protein obtained using either guanidine hydrochloride (a charged denaturant) or urea (an uncharged denaturant) also is dependent on the contribution of electrostatic interactions to protein stability (77).

Increasing the buffer (acetate or phosphate) concentration from 50 mM to 1 M caused a 3- and 10-fold increase in the thermal stability of *P. amagasakiense* glucose oxidase (pI ~4.4) at pH 6.0 and 8.0, respectively, and a 100-fold stabilization at pH 7.0. The thermal stability also was enhanced by 1 M $(NH_4)_2SO_4$, which stabilized the enzyme 100–300 fold at 50 °C and pH 7–8, and 2 M potassium formate (KF), which stabilized the enzyme as much as 36 fold at 60 °C at pH 6.0. In all instances, the deglycosylated enzyme was stabilized to a lesser extent than the native enzyme (78).

Another study showed that a similar increase in phosphate buffer concentration (from 25 to 70 mM) increased the rate of reactivation of *Cyanidium caldarium* latent nitrate reductase when incubated at pH 7.5 and 0 °C (79). This result was postulated to occur because of the dissociation of the nitrate reductase–inhibitor complex by an increase in the ionic strength of the buffer.

The aggregation rate of an acidic fibroblast growth factor (pI = 5–6) decreased as the concentration of phosphate buffer was increased at pH 7.4 (80, 81).The extent of stabilization by various phosphorylated anionic polymers was a result of the interaction between the electropositive heparin binding site on the protein and the anion and was proportional to the chain length of the phosphorylated anionic polymer.

The concentration of urea needed to denature a photointermediate of the photoactive yellow protein was greater in citrate than in acetate buffer at pH 5.0 (82). The slope *m* of the plot between the free energy of unfolding ΔG_u and denaturant concentration was lesser in citrate buffer, which suggested that fewer denaturant molecules binded to the protein on denaturation in citrate than in acetate buffer (83).

A recombinant *Aspergillus fumigatus phytase* (pI = 4.7–5.2) demonstrated better thermostability at 65 and 90 °C in acetate than in citrate buffer at pH 5.5. In addition, the enzyme had a greater heat tolerance in the presence of low concentration (10 mM) than in high concentration (200 mM) of either buffer at 65 °C. Because the heat stability of the enzyme originates from its ability to refold completely into the native-like, fully

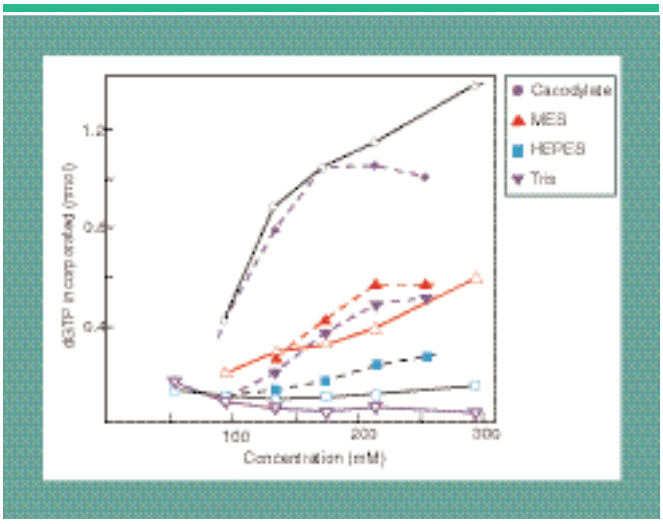

Figure 5: The effect of buffers on the deoxyguanine triphosphate (dGTP) incorporation (adapted from References 85 and 86). Increasing concentrations of KCl were used with a fixed concentration of each of the buffers. For each buffer, dashed lines and solid-colored symbols represent those results.

active conformation after heat denaturation, the results suggested that the refolding was affected by buffer specificity (84).

The relative effectiveness of various buffers at pH 7.2 for the deoxynucleotidyl transferase catalyzed polymerization of the deoxynucleoside triphosphates (dATP, dCTP, and dGTP) onto an oligonucleotide initiator decreased in the following order: cacodylate > MES > HEPES > TRIS > phosphate (85, 86) (see Figure 5). The differences in the effectiveness of the buffers could be attributed neither to differences in ionic strength nor to differences in the amounts of de-protonated buffer ions. The poor effectiveness of the enzyme in a potassium phosphate buffer is most likely a result of the phosphate ion functioning as a competitive inhibitor for the triphosphates.

The half life of L-amino acid oxidase (pI = 4.8) from the Gram-positive bacterium *Rhodococcus opacus* at 37 °C increased more than 20 fold by incubating the enzyme in a glycine-NaOH buffer ($t_{1/2}$ = 938 min) compared with the half life when TEA-HCl ($t_{1/2}$ = 35 min) and a potassium phosphate buffer ($t_{1/2}$ = 44 min) were used (87). The buffer pH was 8.6 for all three buffers. The half life of hydroxynitrile lyase (pI = 4.1) activity decreased in the presence of citrate and acetate buffers at pH 3.75 compared with the half life when phosphate buffer was used (88).

Formulations of $Lys^{B28}Pro^{B29}$ human insulin analog (Humalog, pI = 5.5) comprising TRIS or L-arginine buffer at pH 7.4 remained stable against aggregation for markedly longer periods of time than formulations containing a phosphate buffer (89).

The stability of the α-helical Greek key caspase recruitment domain from the CLARP kinase protein at pH 8.0 (pI = 5.3) decreased in the presence of moderate salt concentrations <200 mM and then exhibited an increase at higher salt concentrations (90). Similar results were obtained for the cold shock protein (Csp) from the thermophilic organism *Bacillus caldolyticus* (91). Results suggested that electrostatic interactions are stabilizing in the native protein, and these favorable coulombic interactions are reduced at low ionic strength. Above the 200-mM salt concentration, the results were consistent with the Hofmeister series. Researchers also demonstrated that the thermostability of Csp increases as the destabilizing effect of salt decreases, probably due to a greater favorable optimization of salt bridges and hydrogen bonds in the thermophilic as compared to the mesophilic species (74, 92, 93).

Similarly, the thermal stability of calf skin collagen type I (pI ~9) in 50-mM acetic acid (pH = 3.0) depended on salt concentration (94). At salt concentrations <20 mM, the salts reduced the denaturation temperature. However, between 20 and 500 mM, they either increased or decreased the denaturation temperature in a salt-specific manner that correlated with their anion position in the Hofmeister series.

The orthophosphate anion HPO_4^{-2} significantly improved not only the thermal stability but also the activity of the endoxylanase (pI = 10.6) at pH 7.0 in 40-mM MOPS buffer. When K_2HPO_4 concentration was increased from 50 mM to 1.2 M, the T_m value of xylanase increased from 60.0 °C to 74.5 °C. The xylanase activity at 0.6-M K_2HPO_4 was 2.3-fold higher than that at 50-mM K_2HPO_4 and 120.2-fold higher than that in 40-mM MOPS buffer. The K^+ cation contributed to the thermal stabilization until 0.6 M, after which the stabilizing effect of the phosphate anion became dominant at K_2HPO_4 concentrations > 0.6 M (95).

Dnase (pI = 3.9–4.3) is a phosphodiesterase capable of hydrolyzing polydeoxyribonucleic acid. Ca^{+2} ion at concentrations >10 mM stabilized the enzyme against aggregation at 37 °C when formulated at pH 6.3, at which the enzyme is stable to deamidation (96, 97). Other divalent cations such as Mn^{+2}, Mg^{+2}, and Zn^{+2} did not stabilize the enzyme. The effect of Ca^{+2} was attributed to specific binding to the active site and preventing aggragation by causing a conformational change in the protein (98).

Phosphate buffer was better than sulfate or imidazole at inhibiting the rate of thermal aggregation and denaturation in β-lactoglobulin (pI = 5.13) at pH 6.7 (99). The researchers speculated that a lysine and arginine-rich region on the edge of the β strands A, E, and F could act as a nucleation center for further unfolding of the protein molecule because of a high surface-charge density. Because arginine and lysine residues can act as sites for phosphate binding, the net charge density is reduced along with the propensity for further unfolding and aggregation (100). Moreover, the net charge on the protein is negative at pH 6.7, and the magnitude of the net coulombic repulsion between the anionic buffer and the protein also can decrease the propensity for denaturation. Anecdotal evidence suggests that the conformational stability of proteins toward denaturation increases if anionic buffers are used above the pI (and conversely, if cationic buffers are used below the pI). This effect is similar to the specific example cited previously and may be viewed as being analogous to the "salting out" effect produced by kosmotropes.

Mobility increments of a 20-mer phosphodiester oligonucleotide were compared for a Tris buffer and various Group I monovalent cations. Organic amines such as TRIS and several Good's buffers bind to the DNA not only by means of electro-

static interactions but also by hydrogen bonds primarily to the purine or pyrimidine rings (101).

Remarkable increases in protein stability can be achieved by improving the coulombic interactions among charged groups on the protein surface. When the hyperexposed Asp49 residue of Ribonuclease T1, an acidic protein with a pI value of ~3.5, was substituted with a histidine, the resulting mutant was 1.1 kcal/mol more stable at pH 6.0 than the wild-type enzyme (102). A buffer molecule that would screen this hyperexposed residue could potentially improve enzyme stability. Indeed, results showed that the conformational stability of the protein was almost doubled with the addition of 0.2 M Na_2HPO_4 at pH 7.0 (103). Tetraprotonated spermine and Mg^{+2} also stabilize Rnase T1 by preferential binding to the folded protein (104). As another example, when two residues of the hexameric glutamate dehydrogenase enzyme from the hyperthermophilic organism *Thermococcus litoralis* were altered to increase inter-subunit ion-pair network attractions, the resulting mutant demonstrated a four-fold improvement of stability at 104 °C over the wild-type enzyme (105).

The thermal stability of the central nervous system defective NK-2 homeodomain protein (pI > 8.6) was investigated using differential scanning calorimetry and ellipticity changes at 222 nm. The presence of 50-mM phosphate at pH 7.4 significantly stabilized the protein with T_m increases of ~13 °C with reference to T_m values observed in 50 mM Hepes at pH 7.4. The stabilization by phosphate was attributed to specific binding because the quench of Trp48 produced by such binding could be partially reversed by the addition of a stoichiometric amount of sequence-specific DNA. The presence of as much as 100 mM NaCl increased the stability and reversibility of unfolding transitions in Hepes buffer but not in phosphate buffer at pH 7.4 (106).

Results of a study showed that the extent and rate of denaturation of rabbit muscle F-actin (pI = 5.4) in the presence of both 50- and 500-mM KCl was increased to a greater extent in MES-NaOH buffer than in a sodium phosphate buffer at pH 6.0 (107).

Another study revealed that chloride salts of choline, Na^+, K^+, Ca^{+2}, and Mg^{+2} increased the stability of the acidic protein apoflavodoxin (pI = 4.0) at neutral pH. The denaturation concentration and free energy of unfolding at constant ionic strength were significantly lower for the protein without added salt and for the protein with the bulky choline cation added in comparison with those with the rest of the cations. The cation stabilizing effect extended to 500 mM, and the researchers speculated that the unusual increase in stability at neutral pH was likely to be a common property among highly acidic proteins (108).

Interleukin 1b (pI = 6.8) solutions were more stable in Tris than in acetate buffer at pH 5.2 at 60 °C (109). At this temperature, the primary degradation pathway was aggregation resulting from the auto-oxidation of cysteine residues. The contribution of the effect of Tris to scavenge hydroxyl radicals was not measured in this study.

The heat-induced denaturation of the recombinant human megakaryocyte growth and development factor (rHuMGDF, pI = 10.7) was partially reversible in Tris and imidazole buffers but not in phosphate or citrate buffers over a pH range of 6.0–8.6 (110). The denaturation was measured using circular dichroism. A correlation also was observed between the reversibility of thermal unfolding—but not the melting temperature itself—and the amount of monomeric protein remaining in solution after storage for two weeks at 37 °C. The surface tension of rHuMGDF was measured as a function of temperature in the presence and absence of sucrose. Unlike Interleukin-1ra, rHuMGDF showed no sharp decrease in surface pressure during melting, thereby suggesting a negligible increase in surface activity and hence a much smaller change in the surface area or volume of the protein upon unfolding. Sucrose consequently had a stabilizing effect on the thermal stability of Interleukin-1ra but not on rHuMGDF.

The thermal denaturation of a recombinant human interferon γ (rHuIγ, pI = 10.3) was studied from pH 2 to 10 (acetate < pH 6.0, phosphate > pH 6.0) and buffer concentration in the range from 5 to 100 mM by differential scanning calorimetry, circular dichroism, fluorescence, and biological activity measurements (111). The thermal transitions were irreversible at all pH values for buffer concentrations of 50 and 100 mM. The transitions were reversible between pH 3.5 and 5.4 at the lower buffer concentrations of 5, 10, and 20 mM. The denaturation enthalpy was directly proportional to the buffer concentration and the denaturation temperature. The sharp decrease in the change in heat capacity with an increase in buffer concentration was attributed to a decrease in the number of hydrophobic groups that were exposed to the buffer by thermal denaturation as a result of preferential aggregation, thereby causing lower stability of the protein at higher buffer concentrations.

Guanidine hydrochloride (GdmCl)–induced unfolding of the yeast prion protein Ure2p (pI = 6.4) was studied in phosphate and Tris buffers (50 mM, 150 mM NaCl) at pH 7.0–8.5 by following the changes in intrinsic tryptophan fluorescence, far UV circular dichroism at 222 nm and 1-anilino-naphthalene-8-sulphonate (ANS) binding fluorescence (112). A three-state denaturation profile was observed: native, dimeric intermediate, and unfolded. In Tris buffer, the native state was stabilized relative to the intermediate, and the profile switched from a three to a two state with a reduction in the range of GdmCl concentrations in which the dimeric intermediate state was populated. In contrast, the free energy required to proceed from the dimeric intermediate to the unfolded state was similar in both buffers. The lag time for amyloid formation was increased in Tris buffer. The buffer effect was a function of the Tris molecule rather than of the phosphate, sodium, potassium, or chloride ions.

The inactivation rate of β-galactosidase (pI = 4.6) was studied as a function of phosphate buffer (pH 7.4) concentration (113). The rate increased until 500 mM and then decreased thereafter until 900 mM. This decrease in the inactivation rate at the higher buffer concentrations was attributed to a decrease in water mobility at these concentrations as measured by its spin-lattice relaxation time using ^{17}O NMR. In another related study, the activity of β-galactosidase was compared using a variety of buffers including four families of Good's buffers. The activity was lowest in phosphate buffer within the 7.0–8.5 pH range (114).

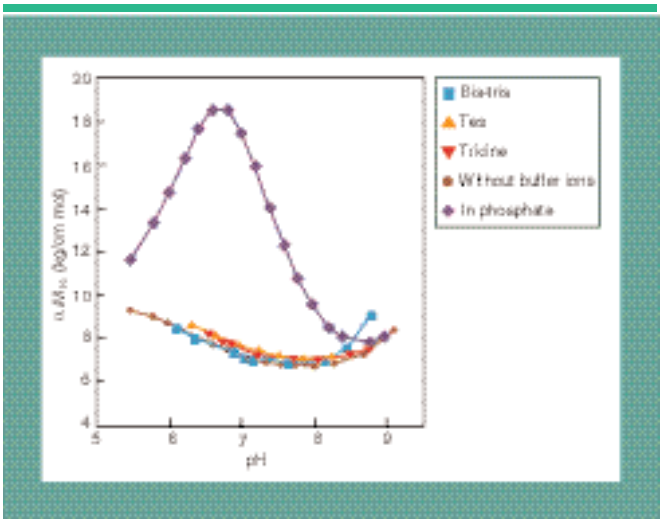

Figure 6: Ultrasonic absorption of oxyhemoglobin as a function of pH (adapted from Reference 125).

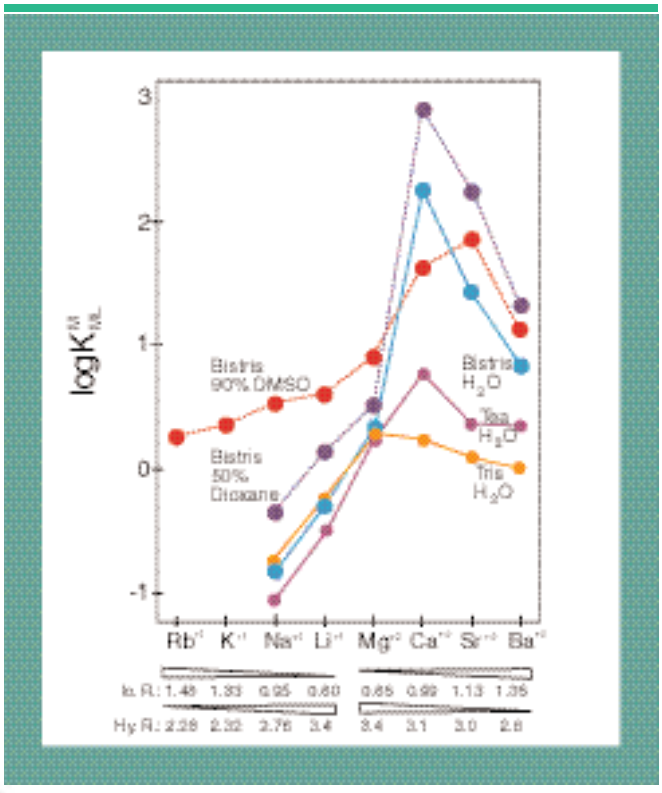

Figure 7: Stability constancts of metal-ion complexes with organic buffers (adapted from Reference 128).

The isoelectric points of aspartate transcarbamoylase and its two mutants, C109H and E119 D, were similar in 10-mM MES buffer. In 10-mM potassium phosphate, the mutants precipitated maximally at pH values 5.9–6.0, distinct from that for the wild type at pH 5.7 (115). The researchers interpreted the phenomenon to mean that the nature of the buffer could influence the conformation of the enzyme mutants.

Results of a study revealed that imidazole-H^+ but not tris-H^+ could replace Na^+ as an activator of ATP-dependent phosphorylation of ATPase. This was achieved primarily by changing the conformation of the enzyme to one that had a high affinity for the ligands participating in phosphorylation (116, 117). Another study involving a more rigorous treatment examined the contribution of the ligand (salt, denaturant) to the free energy of unfolding in terms of the thermodynamics of weak binding systems (i.e., in terms of preferential interactions) (118). A preferential interaction parameter expresses the mutual perturbations of the chemical potentials of the protein and ligand by each other. The perturbation of the chemical potentials leads to a redistribution of solvent components in the domain of the protein.

Effect on proton exchange rates

The degree to which internal protein residues are accessible for hydrogen exchange is inversely related to the global conformational stability of the fluctuating protein ensemble (119). Therefore, in general, conformational stabilization of protein structures is expected to decrease proton exchange rates (120,121). Such exchange rates and consequent protein conformational stability can be readily measured using ultrasound absorption (122–124). As a first approximation, buffers that do not increase the proton exchange rate should be nondestabilizing toward the native conformational structure.

The ultrasonic absorption (i.e., proton exchange rate) of oxyhemoglobin at 1.88 MHz was examined as a function of pH in the presence of phosphate and several Good's buffers (100 mM, see Figure 6) (125). Phosphate buffer resulted in a pronounced increase of protein sound absorption that was attributed to the relaxation processes of proton-transfer reactions between buffer ions and accessible imidazole and α-amino groups and possibly also to the electropositive 2,3-BPG region of the protein surface (58). In contrast, the study showed little absorption for Good's buffers owing to a small reaction volume resulting for a proton transfer between donating and accepting groups.

Effect of buffer–metal complexation

As a result of the formation of ion complexes with metals, buffers can alter protein conformation if the metal of interest acts as a substrate for an enzyme, acts as a catalyst in redox reactions, or changes (usually increases) the free energy of denaturation (126). Of the 20 known Good's buffers, all but three form metal ion complexes.

The stability constants for various aminoalcoholic buffers with alkali and alkali earth metals have been determined in water and mixed aqueous solvent mixtures (127) (see Figure 7). The stability of the alkali ion complexes increased with decreasing ionic radii in aqueous solutions. The stability constant depended on the water activity of any given metal, thereby implicating the differential coordinating abilities of various buffers toward metal ions at protein surfaces (where the activity of water is reduced). Studies showed that the hydroxy groups of aminoalcoholic buffers formed complex with alkali and alkaline earth metals. Therefore, the researchers speculated that corresponding complexes also would be formed with sugars and sugar derivatives.

The use of metal-complexing buffers glycolate, lactate, and

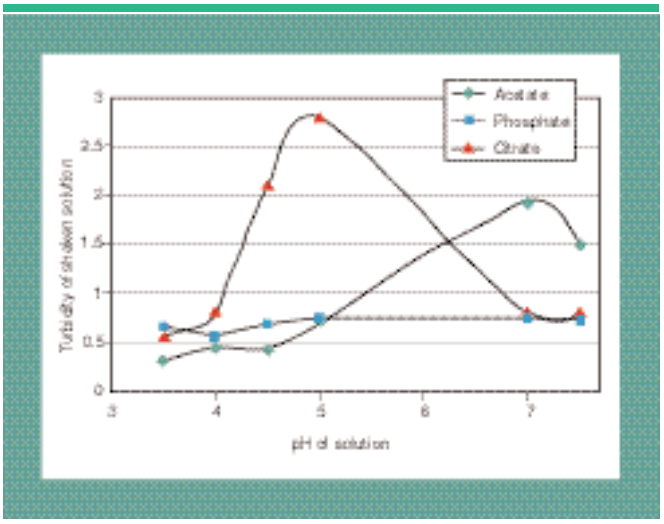

Figure 8: The stabilization of G-CSF solutions against agglomeration in various buffer systems (adapted from Reference 133).

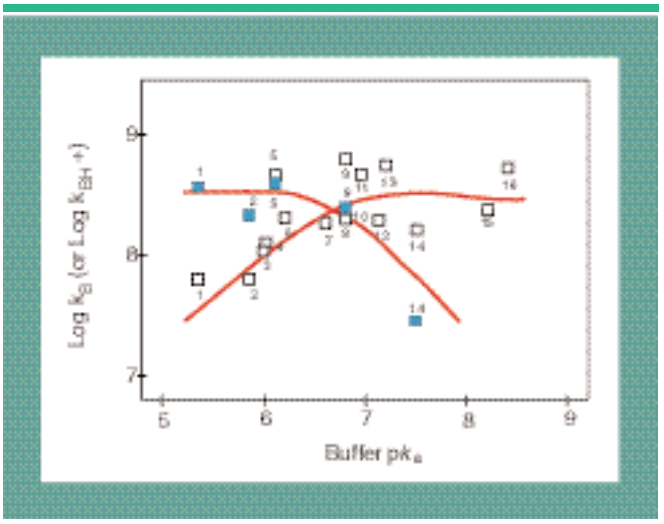

Figure 9: The relationship of proton transfer between an enzyme external buffer and pK_a difference between donor and acceptor buffer species (adapted from reference 135). Figure shows Brönsted plots for rate-limiting proton transfer in CO2 hydration and HCO_3^- dehydration between enzyme and external buffer. The following buffers are represented: (1) malonate, (2) 3-picoline, (3) 2-picoline, (4) 4-picoline, (5) Mes, (6) 3,5-lutidine, (7) 3,4-lutidine, (8) 2,4-lutidine, (9) Aces, (10) phosphate, (11) imidazole, (12) diethylmalonate, (13) N-methylimidazole, (14) Hepes, (15) 1,2-dimethylimidazole, and (16) TAPS.

malonate (50 mM, pH 4.5) increases the manganese peroxide (MnP, pI = 3.2) mediated oxidation of lignin in comparison with the use of acetate buffer (128). The dicarboxylic acid metal-chelating buffers increased MnP turnover by facilitating the dissociation of the oxidized product Mn^{+3} from the enzyme (129). Furthermore, such buffers also stabilized Mn^{+3} in aqueous solution so that it could function as a diffusible mediator oxidizing substrates such as lignin at a distance from the enzyme.

The rate of the reaction of the thiol-specific reagent dithionitrobenzoate (DTNB) with Cys-18 of the silver hake parvalbumin was investigated in various buffers at pH 8.0 (130). A smaller amount of nitrothiobenzoate (NTB) was formed as the buffer was changed from piperazine (~100% positively charged) to Tris (~50% positively charged) to ammonium carbonate (100% negatively charged, least NTB formation). This effect occurred because the metal ion–sequestering capability of ammonium bicarbonate, Tris, and piperazine decreased in that order, thereby leading to a decrease in metal ion–catalyzed oxidation of Cys-18 and, consequently, more Cys-18 being available for interaction with the DTNB reagent.

Another study involved the synthesis of a series of tertiary amine compounds having N-substituents that were ethyl or larger. Results showed that these compounds were stearically inaccessible for bond formation with solvated metal ions in aqueous solution and were capable of functioning as buffers in the pH range 3–11 (131).

Buffer complexation to carbohydrates

The destabilization of β-galactosidase (pI = 4.6) by water miscible organic solvents was studied in borate, phosphate–citrate, and phosphate buffers at pH 7.0 and 8.0. The enzyme showed a marked loss in activity in the borate buffer at pH 8.0 relative to the activity in the borate or the citrate–phosphate buffers at pH 7.0 and the phosphate buffer at pH 8.0 at a 10% solvent composition. The addition of polyhydroxy sugars reduced the enzyme loss only in borate buffer. Researchers postulated that borate ion complexation with the carbohydrates in the glycosylated enzyme made the enzyme more vulnerable to denaturation by organic solvents by changing its conformation. Binding of the borate ions to the polyhydroxy compound prevented or reversed this destabilization effect (132).

Protection against aggregation caused by mechanical stress

The stability of G-CSF toward agglomeration was measured by light scattering at 360 nm over a range of pH values in three different buffer solutions (80 mM) (133). The stabilization of G-CSF against denaturation induced by mechanical stress differes depending on buffer type and pH (see Figure 8). Good correlation was found between the degree of freeze-induced denaturation and that of artificially surface-induced denaturation so that it is unlikely that any surface-active properties of the buffer ions are operative (134).

Degree of buffer protonation: effect of buffer pK_a

The zinc-containing enzyme carbonic anhydrase is an efficient catalyst for the interconversion between CO_2 and HCO_3^-. The rate of proton transfer between external buffer and enzyme depended on the pK_a difference between the donor and the acceptor species in a manner consistent with the presence of a proton shuttle group (His-64) in the enzyme of $pK_a \approx pK_b \approx 7$ (see Figure 9) (135). Although the buffers do not appear to cause a change in the conformation of the enzyme, researchers have suggested that any proton transfer event associated with allosteric or catalytic enzyme sites is associated with a partial denaturation that is cooperative within considerable but localized regions of the protein domain (136, 137). In addition, a quali-

tative agreement appears to exist between the number of extra water molecules that are thermodynamically bound upon denaturation and the number of water molecules implicated in some ligand-binding processes (138). If these numbers reflect the magnitude of the conformational changes involved, then it follows that considerable three-dimensional changes must take place in the protein structure upon ligand binding.

In another study, the Gln-64 and Ala-64 mutants of the enzyme yielded significantly higher K_{cat} values in imidazole, CHES, and phosphate buffers than in Bicine, TAPS, and MOPS buffers. The researchers attributed this effect to the ability of the former three buffers to function as efficient proton-transfer groups instead of Lys-64 (in the wild-type enzyme) while the latter three buffers lacked this ability (139).

The binding of EcoRV, a Type II restriction enzyme, to pBR322 plasmid DNA was measured in various amine-based (Tris, Bis-Tris propane, HEPES, and TES) buffers (140). Results revealed that the pK_a dependent extent of protonation of the amine buffer correlated with K_m. The correlation was poorer in amine buffers with a "buried" and stearically less-accessible positive charge. These results were attributed to the protonated amines of the pH buffer acting as counter-ions to the DNA phosphate, thereby modifying the binding of enzyme to DNA.

In another study, preincubation of Lipofectin in 30–80 mM phosphate buffer at pH 5.6–6.8 resulted in as much as 26–56-fold increases in luciferase expression from plasmid DNA and mRNA, respectively (141). The increased transfection was specific to the divalent phosphate ion in that monovalent Cl^- and CH_3COO^- ions were not stimulatory and phosphate buffer at pH $>$ 7.6 caused a sharp drop in transfection efficiency. Researchers speculated that nucleic acids in the presence of divalent phosphate anion were more efficiently encapsulated to a condensed structure, more easily able to cross cell and endosomal membranes, and become readily translatable, thereby resulting in superior nonviral gene transfection efficiency.

The inhibitory effect of phosphate buffer on jack bean urease (pI 5 5.1) at 362 mM and pH 7.5 was less than that of the buffer at 0.5 mM and 5.8 pH. The inhibition paralleled the concentration of the $H_2PO_4^-$ ion. The deprotonation of the histidine at the active site of the enzyme at pH $>$6.5 resulted in a repulsion of the $H_2PO_4^-$ ion, thereby resulting in a progressive decrease in its inhibitory effect (142).

Substrate substitution effects

The structure of *R. marinus* hyperthermostable cellulase Cel12A was compared with the structure of mesophilic *S.lividans* CelB2. CelB2 was crystallized with (2nlr) or without (1nlr) a substrate inhibitor (2-deoxy-2-fluorocellotrioside), and Cel12A was crystallized from 100 mM HEPES buffer. The crystal structure of Cel12A was more silimar to the active conformation of CelB2(2nlr) than to the native unliganded CelB2(1nlr). Results showed that a HEPES buffer molecule was bound in the active site of Cel12A and this effect triggered a conformational change to an active configuration. The researchers concluded that the active state conformation of the Cel12A enzyme is induced by the presence of HEPES (143). Another study showed that the proportion of low- or high-affinity ester hydrolyzing antibody conformers raised against a phosphonate transition state analog could be altered by changing the buffer used for crystallization (29).

Annexin V undergoes significant changes in domain III when crystallized in the presence of a high concentration of calcium, thereby resulting in the formation of a new calcium site by displacement of Trp187 from a buried to an exposed conformation (144). It is possible that the lyophilization of a protein from a buffer or excipient can alter its conformation such that such an altered conformation may then subsequently become more (less) susceptible to denaturation/aggregation when exposed to a subcutaneous or intravenous environment.

Values for the Michaelis constant (K_m) for the Mg_2PP_I hydrolysis by *E. coli* inorganic pyrophosphatase (E-Ppase) were smaller and similar in different zwitterionic buffers (TES, MOPS) but larger and more varied in various mono-aminoalcoholic buffers (e.g., TRIS, 2-amino-2-methyl-1,3-propanediol, monoethanolamine) (145). Researchers hypothesized that the aminoalcohols interacted with the substrate binding site of E-Ppase and stabilized the enzyme hexamer. They indeed found that the positions of the oxygen atoms and the positively charged groups in 2-amino-2-methyl-1,3-propanediol and the substrate magnesium pyrophosphate were superimposible within 0.4 Å The monoamino group and monoaminoalcoholic buffers should hence be a good substitute for Mg^{+2}. Quaternary ammonium ions can substitute for Mg^{+2} in bacteriorhodopsin with maintenance of proton pumping, and polyamines can partially substitute for Mg^{+2} as activator of *Streptococcus faecalis* Ppase (146, 147).

Effect on protein-surfactant interactions

Buffers can alter protein-surfactant binding characteristics and thereby change protein conformation. Results of a study showed that increasing the concentration of sodium phosphate buffer (pH = 7.1) from 10 to 100 mM increased the amount of sodium dodecyl sulfate (SDS) bound to reduced-carboxyamidomethylated bovine serum albumin (RCAM-BSA) from 1.0 to 2.2 g/g (148).

In another study, a coadsorbed multilayer of SDS and lysozyme formed in the transitional binding regime at pH 6.9 in 8.8 mM phosphate buffer but not at pH 5.0 in 5.0 mM acetate buffer (149). The binding isotherms showed that approximately the same number of molecules of SDS bound to lysozyme between the onset and completion of transitional binding at both pH values. The greater aggregation tendency in the phosphate buffer was speculated to be caused by a more effective charge screening by the divalent HPO_4^{-2} than by the univalent CH_3COO^- ions.

Conclusion

Historically thought of as innocuous substances, buffers have profound effects on the tertiary and quaternary structures of proteins. It is important to realize that buffers perturb protein conformational stability because of a complex interplay between various effects rather than by stand-alone mechanisms. For example, some of the antioxidant effects of Good's buffers may arise because of their metal binding ability (150). Binding or substrate effects may predominate the interaction of buffers with proteins at low buffer concentrations (80, 87); electrostatic charge screening may dominate at intermediate concentra-

Table II: Summary of physicochemical and structural properties of proteins in various buffer systems.

pH, pI, charge	Molecule	Folding*	Mechanism of stabilization	Buffer
6.9, 8.3, positive	Organophosphorous hydrolase	E, α/β	Kosmotrope/chaotrope, CHES poorer	Phosphate
6.2, 6.0, negative	Phosphoenol pyruvate carboxylase	E, α/β	Kosmotrope/chaotrope	Tetramer
5.5, (4.7–5.2), negative	*Aspergillus fumigatus* phytase	E, α/β	Citrate poorer	Acetate
7.5, 4.6, negative	β-galactosidase	E, β	Phosphate poorer	Good buffers
8.0, 5.3, negative	CLARP	N, β	Decrease in stability until 200 mM then increase, buffer used was 30 mM TRIS	
6.7, 5.1, negative	β-lactoglobulin	N, β	Sulfate, imidazole poorer buffers, phosphate specific binding	Phosphate
7.0, 3.5, negative	Rnase T1	E, α + β	Na_2HPO_4, spermine and Mg^{+2} stabilize by preferential binding	Phosphate
7.4, >8.6, positive	Homeodomain	N, α	HEPES poorer; specific binding by phosphate	Phosphate
(6.0, 8.0), 4.4, negative	Glucose oxidase	E, α/β	Binding, stability also increased by $(NH_4)_2SO_4$ and KF. Increasing buffer concentration increases stability.	Phosphate, acetate
7.0, 6.9, negative	Hemoglobin	N, α	Phosphate decreases stability as a result of binding to 2,3-BPG site	HEPES, TRIS
7.0, 4.0, negative	Apoflavodoxin	N, α/β	Binding stability increase until 500 mM	
7.0, 10.6, positive	Endoxylanase	E, β	MOPS not optimal buffer, K_2HPO_4 improved thermal stability and activity	Phosphate, K+
3.0, 9.0, positive	Collagen Type I	N, NOS	Binding, stability decrease until 20 mM then increase until 500 mM	
7.4, (5–6), negative	Acidic fibroblast growth factor	N, β	Binding to electropositive heparin binding site; phosphorylated anionic polymers increase stability	Phosphate
(6.0, 8.6), 10.7,	RHuMGDF	N,U	Cationic buffers increase stability below pI; phosphate citrate poorer	TRIS, imidazole
6.3 (3.9–4.3), negative	Dnase	E, α + β	Binding (Ca^{+2}) ion-specific increases stability	
7.4, 5.5, negative	LisPro human insulin analog	N, NOS	Stability decreased in phosphate buffer	TRIS or L-arginine
5.2, 6.8, positive	Interleukin 1β	N, β	Stability decreased in acetate buffer, TRIS inhibits auto-oxidation?	TRIS
(7.0, 8.5), 6.4, negative	Yeast prion protein, Ure2p	N, α	Phosphate poorer buffer. TRIS increases free energy of transition from native to dimeric intermediate.	TRIS
(2,10), 10.3, positive	rHuIγ, interferon γ	N, α	Increasing the concentration of phosphate or acetate buffer causes increased agglomeration	N/A
8.6, 4.8, negative	L-amino acid oxidase (AAO)	E, α/β	Stability poorer in TEA-HCl or potassium phosphate buffers.	Glycine-NaOH
4.5, 3.2, negative	Manganese peroxidase (MnP)	E, α	Acetate poorer buffer; enzyme activity increased in metal-chelating buffers.	Glycolate, lactate, malonate
5.0, U, U	Photoactive yellow protein (PYP)	N, α/β	Acetate poorer buffer.	Citrate
U, U, U	Inorganic pyro-phosphatase	E, β	Aminoalcoholic buffers poorer due to acting as substrate mimics	TES, MOPS
3.75, 4.1, positive	Hydroxynitrile lyase	E, α/β	Citrate and acetate poorer	Phosphate
6.0, 5.4, Negative	F-actin	N, α/β	MES poorer buffer	Phosphate

*N denotes proteins without enzymatic function; E denotes enzyme; α denotes lower-order fold configuration; β, α + β, α/β denote higher order configurations; U denotes unkown; and NOS denotes no ordered structure.

tions (64, 82); and kosmotropic/chaotropic effects may prevail at higher concentrations (43, 73). The contribution of charge repulsion by buffer anions to thiol–disulfide exchange reactions may vary with the degree of buffer deprotonation (90, 99), as can the contribution of buffer to amide exchange rates (151).

Because of the extremely diverse structure and related properties of proteins, it may not be possible to predict a priori the "best" buffer for any given protein molecule. However, some correlative generalizations can be attempted—recognizing that these may not necessarily be causative in nature. Buffers that may best protect a given protein from a variety of denaturing stresses should possess the following attributes:

- ability to incorporate the electron-donating and electron-accepting sites on one molecule (i.e., be zwitterionic)
- preferentially be excluded from the protein domain (i.e., increase the surface tension of water) and incorporate kosmotropic ions
- scavenge free radicals
- possess a low heat of ionization
- decrease the mobility of water molecules
- cause negligible change in the denaturation Gibbs energy (ΔG_d), for that protein
- not undergo or catalyze complexation with the carbohydrate part of the glycosylated protein
- inhibit the nucleophilic attack of the thiolate anion on disulfide links, thus preventing thiol–disulfide interchange.
- unless intended, not act as a substrate for the enzyme, not catalyze metal mediated redox reactions or alter surfactant binding characteristics to the protein
- not render the protein more susceptible to mechanical stress
- not cause an increase in the proton amide exchange rate for the protein residues with the buffer vis-a-vis an "inert" buffer medium.

Table II summarizes the physicochemical properties of proteins and enzymes in various buffer systems (as referenced in this article) and their corresponding structural configurations. The folding configurations were obtained from the Structural Classification of Proteins database on the Internet (release 1.63, 2003) (151, 152).

Figure 10 shows points at which phosphate is a good buffering agent (shown in red) and those at which it is a poor buffer (in blue) under the conditions used in each study (from Table II). The graph was plotted by assigning the numbers 1 and 2 for enzyme and nonenzyme, respectively, along the *x–z* axis; the numbers 0,1, 2, 3, and 4 for no-ordered structure, α, β, α + β, and α/β folds, respectively, along the *x–y* axis; and the difference between the pH of the study and the isoionic point pI of the protein along the *y–z* axis.

From the figure, it appears that phosphate is an equally good buffer for both proteins (4/9 or 4 out of 9 proteins) and for enzymes (5/9) at higher fold configurations (i.e., β, α + β, and α/β [8/9]), but it is a poorer buffer for proteins at the lower fold configurations (i.e., no-ordered structure or α folds [5/7]). Although exceptions exist, such approaches may lend themselves to a better understanding of why certain buffers may stabilize conformation of certain proteins and destabilize others. Phosphate was chosen for no other reason other than because the greatest number of points could be obtained using this buffer.

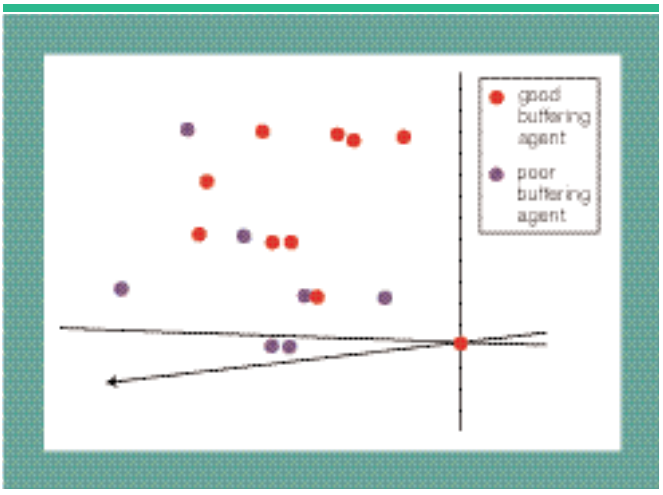

Figure 10: A plot of folding configuration versus difference between pH and pI for several enzymes and proteins.

Figure 10 should be viewed with many caveats. In some instances, only two buffers were compared. Therefore, this does not preclude the possiblity that had more buffers been included in the study, the outcome may have been different from what was observed. The buffer effect may have possibly contributed significantly toward the detector response. In other words, the results may not be indicative of the buffer response on the system but rather on its response on the means of measurement. For example, the buffer effect on the photoelectrochemical response of bacteriorhodopsin was a result of suppression of interfacial pH and not a result of any specific effects on proton transfer after photoisomerization of the retinal chromophore (153).

The method used for protein extraction and purification may affect how a particular buffer subsequently modulates its stability. For example, solubilization of the terminase enzyme from inclusion bodies with either sarkosyl ($gpNul_{srk}$) or guanidine hydrochloride ($gpNul_{gdn}$) with subsequent purification resulted in $gpNul_{srk}$ being more stable to thermally induced or guanidine hydrochloride–induced denaturation than $gpNul_{gdn}$ in pH 8.0 imidazole buffer (154). A change in protein conformation or stability induced by a buffer can vary with buffer pH, thereby resulting in the possibility that a buffer may become poorer (or better) at a different pH (135, 142). Finally, it should be obvious that the subset of proteins represented here is part of a much larger protein population and the results may not necessarily be extrapolable to the entire set of proteins.

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