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# Ultrasonic absorption studies of protein-buffer interactions

## Determination of equilibrium parameters of titratable groups

**K. D. Jürgens* and R. Baumann**

Zentrum Physiologie, Medizinische Hochschule Hannover, Konstanty-Gutschow-Strasse 8, D-3000 Hannover 61, Federal Republic of Germany

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**Abstract.** The acoustic absorption of protein solutions in the presence of phosphate and other buffering ions has been studied in the physiological pH range. Buffers containing hydroxyl residues as titratable groups cause a pronounced increase of protein sound absorption, which is attributed to relaxation processes of proton transfer reactions between buffer ions and accessible imidazole and α-amino groups of the protein surface. Amino group based buffers like Good's buffers do not induce additional sound absorption. Measurement of the ultrasonic absorption as a function of pH and of buffer concentration, and corresponding parameter fitting of the equation describing proton transfer relaxation processes has been used to evaluate equilibrium parameters. For the imidazole group of the amino acid histidine a pK value of 6.22 and for the imidazole group of the protein lysozyme a pK value of 5.71 have been determined. In hemoglobin the ligand-linked pK changes have been monitored by recording ultrasonic titration curves.

**Key words:** Ultrasonic absorption, equilibrium constants, histidine, lysozyme, hemoglobin

### Introduction

In a previous investigation of the ligand-linked ultrasonic absorption of hemoglobin it was observed that the absorption coefficient of hemoglobin solutions in the neutral pH range was altered drastically in the presence of inorganic phosphates (Jürgens et al. 1980). Excess sound absorption of a protein solution caused by inorganic phosphate has also been observed by Slutsky et al. (1984), who studied ribonuclease. Sound absorbing mechanisms arising from interactions between proteins and phosphate ions are of considerable interest since inorganic phosphates are widely used as buffers in protein chemistry and organic phosphates play a dominant rọle in the regulation of protein function as well as being substrates for enzymes. In the MHz frequency range, chemical relaxation processes are major contributors to the measured sound absorption (Lang et al. 1973). Of particular interest are proton-transfer relaxation processes since sound absorption caused by these processes can be used to determine the equilibrium parameters of titratable groups. A general description of dynamic titration methods has been outlined by Winkler-Oswatitsch and Eigen (1979). In the present study we have developed a model for proton transfer processes involving protein side chains and buffer anions, which allows the calculation of the pK values, reaction rate constants, and reaction volumes from the pH and concentration dependence of the ultrasonic absorption. The method has been applied to evaluate the systems histidine-phosphate and lysozyme-phosphate, and to show oxygen-linked pK changes of hemoglobin side chains.

### Experimental

Preparation of hemoglobin solutions, deoxygenation, and determination of protein concentration and oxygen content was performed as described previously (Jürgens et al. 1980). Aqueous solutions of egg white lysozyme (Serva), bovine milk β-lactalbumin (Sigma), equine skeletal muscle myoglobin (Sigma), bovine serum albumin (Merck), and hemoglobin were prepared with a chloride concentration of 0.1 *M*. All buffer solutions were adjusted to the initial pH value of the protein solutions before being added. This was done for secondary phosphate by mixing appropriate amounts of di-sodium hydrogen phosphate and potassium dihydrogen

* To whom offprint requests should be sent

phosphate (Merck). Pyrophosphate and phosphite were prepared by titrating diphosphoric acid or phosphorous acid (Riedel de Haen), respectively, with 0.1 *M* NaOH. *Bis tris* buffer (Sigma) and the zwitterionic buffers Tes (N-*tris*(hydroxymethyl)-methyl-2-aminoethanesulfonic acid) and Tricine (N-(2-hydroxy-1,1-*bis*(hydroxymethyl)ethyl)glycine) (Promochem) were titrated with 0.1 *M* HCl or 0.1 *M* NaOH, respectively. After adding the buffer solutions, the ultrasonic titration curves were recorded by an ultrasonic absorption measuring apparatus described previously (Jürgens et al. 1980).

## Results and discussion

As has been shown by Jürgens et al. (1980) the specific ultrasonic absorption coefficient, $\alpha/m_{Hb}$ ($m_{Hb}$ – molal concentration of hemoglobin tetramer $Hb_4$), of hemoglobin solutions increases at 1.88 MHz in the presence of inorganic phosphate ($P_i$) at a molar ratio of 10 : 1 ($P_i$ : Hb) up to twice the value of phosphate-free hemoglobin solution of the same protein concentration. Ultrasonic absorption arising from phosphate-phosphate interactions can be neglected since we found that protein-free solutions of inorganic phosphate of comparable concentration exhibit only a very small sound absorption.

The sound absorption of intact and urea-denatured proteins has been investigated by Lang et al. (1971), who studied lysozyme, $\beta$-lactoglobulin, and bovine serum albumin. They found absorption peaks in the native as well as in the denatured state and concluded that the sound absorbing relaxation processes are caused by dissociative proton transfer reactions, in the lower pH range occurring between hydrogen ions and acid amino acids, and in the upper pH range between hydroxyl ions and alkaline amino acids. In the neutral pH range, where the phosphate-induced sound absorption occurs, direct proton transfer reactions between proton donating and proton accepting molecules can be the reason for considerable sound absorption. The buffering phosphate ions can interact with appropriate amino acid residues such as the imidazole groups of histidines and free $\alpha$-amino groups on the surface of the protein (Slutsky et al. 1980, 1984). The proton transfer reactions would proceed according to the following reaction scheme:

$$\begin{array}{ccccc} H^+ + HPO_4^{2-} + {=}N- + H_2O & & & & \\ {\scriptstyle k_{31}}\swarrow\nearrow{\scriptstyle k_{13}} & & & & {\scriptstyle k_{3'1}}\searrow\nwarrow{\scriptstyle k_{13'}} \\ H_2PO_4^- + {=}N- + H_2O & \underset{k_b}{\overset{k_f}{\rightleftharpoons}} & HPO_4^{2-} + {=}\overset{N-}{H+} + H_2O & & \\ {\scriptstyle k_{32}}\searrow\nwarrow{\scriptstyle k_{23}} & & {\scriptstyle k_{23'}}\swarrow\nearrow{\scriptstyle k_{3'2}} & & \\ H_2PO_4^- + {=}\overset{N-}{H+} + OH^- & & & & \end{array}$$

In the neutral pH range the concentration of hydrogen and hydroxyl ions ($c_H$, $c_{OH}$) is very small compared to the concentration of phosphate and imidazole or $\alpha$-amino groups ($c_{HPO_4^{2-}}$, $c_{H_2PO_4^-}$, $c_N$, $c_{NH^+}$). Therefore, only the direct transfer between phosphate and protein contributes significantly to the sound absorption (Eigen 1964). At a pH around 7 the reciprocal relaxation time for this bimolecular process is

$$1/\tau = k_f(\bar{c}_{H_2PO_4^-} + \bar{c}_N) + k_b(\bar{c}_{HPO_4^{2-}} + \bar{c}_{NH^+}) .$$

Inserting the expressions for the equilibrium constant of the dissociation reaction of phosphate ($K_P$) and of the corresponding reaction partner ($K_N$) as well as the expressions for the total concentrations of phosphate ($c_{Ph}$) and the reaction partner ($c_{Pr}$), leads to the equation for the relaxation frequency

$$f_R = \frac{k_f}{2\pi}\left(c_{Ph}\frac{\bar{c}_H + K_N}{\bar{c}_H + K_P} + c_{Pr}\left(\frac{K_N + \frac{K_N}{K_P}\bar{c}_H}{K_N + \bar{c}_H}\right)\right). \qquad (1)$$

The reaction rate constants, $k_f$ or $k_b$, respectively, are expected to be determined mainly by the diffusion coefficient of the buffer molecule, which in general is much larger than the diffusion coefficient of the protein.

The effect on sound absorption due to reaction enthalpies is negligible compared to the effect due to reaction volumes ($\Delta V$) (Eigen and de Maeyer 1963). Then the specific absorption coefficient is given by,

$$\alpha/c_{Pr} = \frac{v\varrho}{2RT}\,\frac{\Delta V^2 k_f c_{Ph}}{(1 + K_P/\bar{c}_H)(1 + \bar{c}_H/K_N)}\,\frac{f^2}{f_R^2 + f^2}, \qquad (2)$$

where $\varrho$ is the density and $v$ the sound velocity of the solution, $T$ is the temperature, $R$ the gas constant, $f$ the measuring frequency.

If the reaction partner is a protein containing more than one group which can participate in proton transfer with the buffer molecules, the reactions are coupled by the common buffer and a system of coupled equations results. In this case the relaxation frequency spectrum has to be calculated from the corresponding matrix. Only if all $n$ groups have identical pK values and are sterically equally accessible (i.e. they have equal $k_f$ values) the spectrum degenerates to one relaxation frequency. Then $c_{Pr}$ has to be replaced by $n\,c_{Pr}$ in Eq. (1) and the specific absorption coefficient of the protein is $n$ times the value given by Eq. (2).

Calculations of the ultrasonic absorption of single proton transfer relaxation processes, based on this model, have been performed with different donor pK values and a constant acceptor pK of 6.88 for the secondary phosphate at a ionic strength of

0.1 (Fig. 1). They lead to the result that only amino acid side chains with pK values between 5 and 9 contribute significantly to the sound absorption in the neutral pH range. This means, in terms of protein composition, that imidazole groups of histidines and α-amino groups of the terminal valines and to a lesser extent ε-amino, γ-carboxyl, and thiol groups with abnormal pK values are proper partners of the phosphate ions for acoustically relevant proton transfer. Reactions of this type have also been proposed by Slutsky et al. (1980, 1984) as the basis for the excess sound absorption observed in aqueous solutions of the antibiotic bacitracin and of ribonuclease buffered with inorganic phosphate.

To prove the validity of this model, several experiments were carried out varying some parameters affecting the sound absorption. The number of titratable amino acid side chains of the protein molecule ($n$) should have an influence on the absorption coefficient. In fact proteins, which differ in their histidine content show a clear relation between the number of histidines and the magnitude of the sound absorption coefficient. Figure 2 shows the results for lysozyme (1 *His*), β-lactalbumin (3 *His*), myoglobin (11 *His*), serum albumin (17 *His*), and human hemoglobin A (38 *His*). All solutions contained inorganic phosphate in a molar ratio of 10 : 1 ($P_i$ : protein) and had the same molar protein concentration. These results indicate that many imidazole and α-amino groups are probably accessible to phosphate ions and therefore must be regarded as potential contributors to the total phosphate-induced absorption. In the case of hemoglobin up to 22 histidine groups per tetramer are reported to be unmasked (Greenfield and Williams 1972; Ohe and Kajita 1980; Matthew et al. 1979; Russu et al. 1982).

To check the influence of the pK value of the buffer ion on the sound absorption, phosphate and other phosphorous containing compounds were added to solutions of oxy and deoxyhemoglobin. Phosphite, the salt of phosphorous acid, has a pK value of 6.4 (20 °C, $I = 0.1$, Smith and Martell 1976), which is about 0.5 pH units ($\Delta$ pK) lower than that of phosphate. As shown in Fig. 3, the maximum of the ultrasonic titration curve of the hemoglobin-phosphite system is shifted about 0.3 pH units to the acid range compared to the hemoglobin-phosphate curves. This is in good agreement with the theory of proton transfer relaxation processes, predicting a shift of the absorption peak of $\Delta$ pK/2. This shift holds for each single process and, provided that all pK values of the protein groups involved are in a narrow range, holds also for the superimposition of multiple processes. Pyrophosphate, the salt of diphosphoric acid, has pK values at 6.0 and 8.3 (20 °C, $I = 0.1$), (Smith and Martell 1976). The cor-

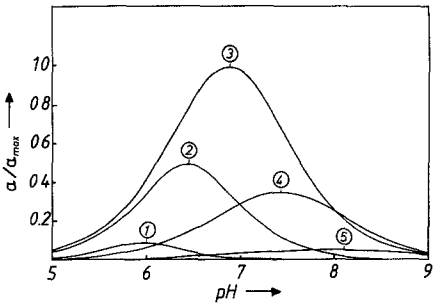


**Fig. 1.** Relative sound absorption of proton-transfer relaxation processes as a function of pH. The pK value of the acceptor group is chosen as 6.88, donor pK values are 5 (*1*), 6 (*2*), 6.88 (*3*), 8 (*4*), and 9 (*5*)

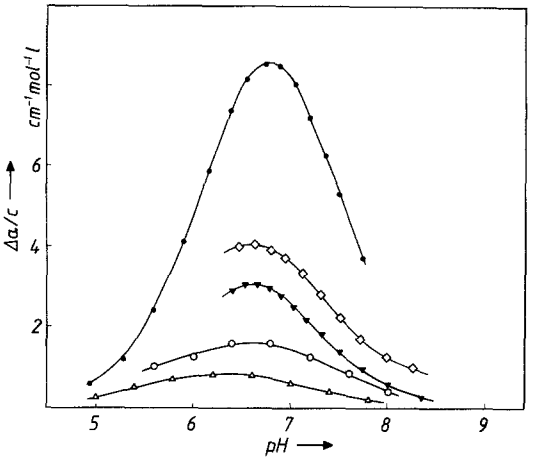


**Fig. 2.** The phosphate-induced specific absorption of proteins with different histidine content as a function of pH. ([protein]: [phosphate] = 1 : 10). Egg white lysozyme (△), bovine β-lactalbumin (○); horse skeletel muscle myoglobin (▼), bovine serum albumin (◇), human oxyhemoglobin (●). $c_{Pr} = 1.5$ m$M$, $f = 1.88$ MHz, $t = 20$ °C, $[Cl^-] = 0.1\,M$

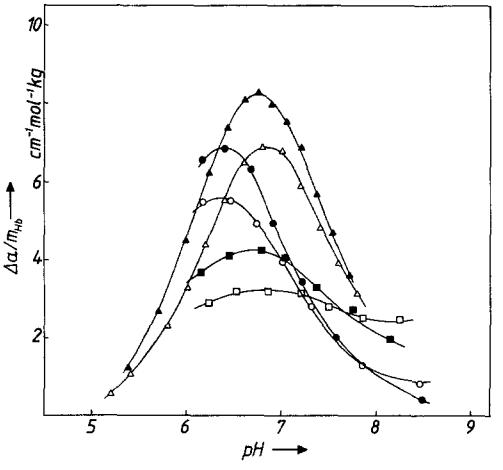


**Fig. 3.** The additional ultrasonic absorption of oxygenated and deoxygenated human hemoglobin caused by a tenfold molar excess of phosphate (▲, △), phosphite (●, ○), and pyrophosphate (■, □), as a function of pH. $m_{Hb} = 1.7$ mmol/kg, $t = 20$ °C, $[Cl^-] = 0.1\,M$, $f = 1.88$ MHz

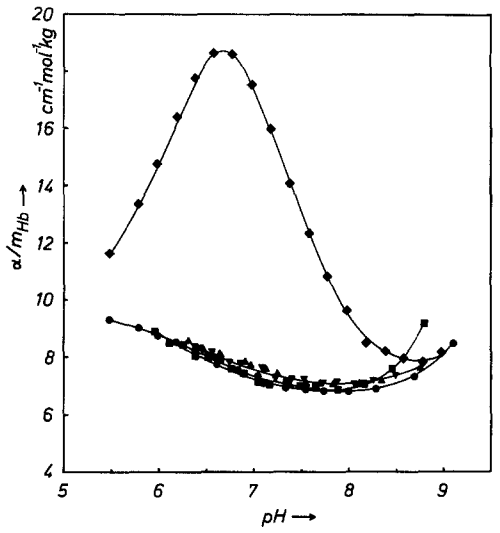


**Fig. 4.** The ultrasonic absorption of oxyhemoglobin as a function of pH in the presence of a tenfold excess of the buffers *Bis-tris* (■), Tes (▲), and Tricine (▼). For comparison the results without buffer ions (●) and in the presence of phosphate (◆) are shown. $m_{Hb} = 1.7$ mmol/kg, $t = 20\,°C$, $f = 1.88$ MHz, $[Cl^-] = 0.1\,M$

responding absorption peak of the hemoglobin-pyrophosphate solution (Fig. 3) is much broader than the one caused by phosphate or phosphite. Obviously, the superimposition of proton transfer processes involving two dissociation steps is reflected here in the sound absorption.

Other buffers with pK values in the neutral range which cause considerable sound absorption due to proton exchange with proteins are hydrogen sulfite (pK = 7.1) and, less pronounced, cacodylate (pK = 6.1). At 1.88 MHz a tenfold excess of buffer ions over hemoglobin ($c_{Hb} = 1.3$ m$M$), hydrogen sulfite causes an absorption increase of similar magnitude to that caused by phosphate, whereas the effect of cacodylate is only 12% that of phosphate. In addition, we tested buffer solutions used in the physiological pH range, such as Tris or Bis-tris (pK 8.2 and 6.57, 20 °C), (Bates 1973) and the buffers recommended by Good et al. (1966) such as Tes or Tricine (pK 7.5 and 8.15, 20 °C). As can be seen from Fig. 4, in comparison to phosphate these ions do not induce a significant increase in absorption upon addition to oxyhemoglobin solutions in the frequency range investigated. In contrast to the negatively charged ions, where a hydroxyl group is protonated or deprotonated, the buffering properties of these substances are based on the dissociation of a hydrogen ion from an amino or imino group. Since the latter is also true for the buffering residues of the protein in the physiological range, probably only a small reaction volume ($\Delta V$) results for a proton transfer between donating and accepting groups, whereas the transfer between hydroxyl and imidazole or α-amino groups is associated with a large $\Delta V$ (Millero 1971; Kauzmann et al. 1962). These results also imply that not all buffers are suitable for investigations of the intrinsic sound absorption of proteins, non-absorbing buffers must be chosen.

After having given convincing evidence for the validity of the model, it was used to determine equilibrium parameters of some biological substances. Before being applied to protein-phosphate interactions, the model has been used to describe a simpler reaction system, the transfer interaction between phosphate and an aqueous solution of the amino acid histidine. In Fig. 5a, b, the pH and concentration dependence of the specific absorption of this system are shown. Assuming a pK of 6.88 for secondary phosphate at 20 °C and $I = 0.1$ (Smith and Martell 1976), a parameter optimization based on evolution strategy (Schwefel 1977) was carried out. The best fit gained from several runs with different starting values led to the results shown in Table 1. All values are in good agreement with data

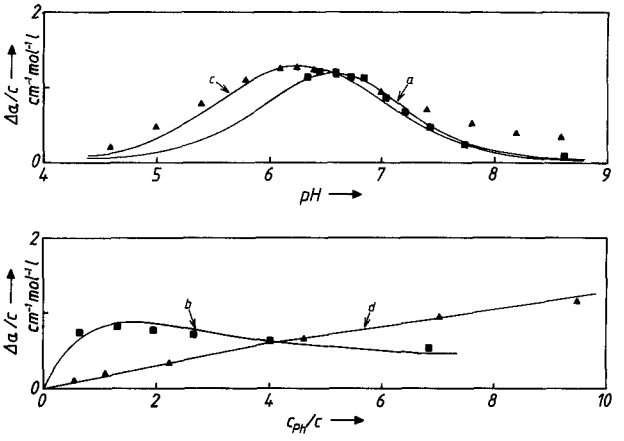


**Fig. 5.** pH and concentration dependence of the phosphate induced specific absorption of histidine (*a*, *b*) and lysozyme (*c*, *d*) solutions (measured values and best fits). $t = 20\,°C$, $f = 1.88$ MHz, $[Cl^-] = 0.1\,M$. (*b*) pH = 7.1, $c_{His} = 10$ m$M$; (*a*) molar ratio 1:1, $c_{His} = 10$ m$M$; (*d*) pH = 6.3, $c_{Lys} = 2.5$ m$M$; (*c*) molar ratio 10:1, $c_{Lys} = 2.5$ m$M$

**Table 1.** Means and standard deviations of reaction rate constants, reaction volumes, and equilibrium constants of some imidazole groups calculated from ultrasonic titration curves

| Substance | $k_f$ $M^{-1}s^{-1}$ | $\Delta V$ ml/mol | pK |
|---|---|---|---|
| Histidine | $(2.3 \pm 0.2)\,10^8$ | $24.7 \pm 0.5$ | $6.22 \pm 0.08$ |
| Lysozyme | $(2.1 \pm 0.4)\,10^7$ | $38.7 \pm 3.5$ | $5.71 \pm 0.09$ |
| Oxyhemoglobin | $(0.9 \pm 0.6)\,10^8$ | $19.5 \pm 2.5$ | $6.30 \pm 0.25$ |
| Deoxyhemoglobin | $(1.2 \pm 0.5)\,10^8$ | $17.3 \pm 3.0$ | $6.50 \pm 0.25$ |

obtained by other methods (Eigen 1964; Kauzmann et al. 1962; Martell and Smith 1976) and with the ultrasonic results of Slutsky et al. (1980) for imidazole-phosphate and bacitracin-phosphate interactions.

The results shown in Fig. 5c, d have been obtained from the pH and concentration dependence of the ultrasonic absorption of the protein-buffer system lysozyme-phosphate. In hen egg white lysozyme only one histidine is present (Dayhoff 1975). For the imidazole group of this histidine the pK value and the other equilibrium parameters derived from the curve fitting procedure are also shown in Table 1. The reaction rate constant and the reaction volume are in the range expected for proton-transfer reactions (Eigen 1964; Kauzmann et al. 1962). The pK value of 5.71 is in excellent agreement with the value of 5.8 obtained from NMR-studies by Meadows et al. (1967), performed at 28 °C and a higher protein concentration as well as with the NMR result of Shindo et al. (1977), who found, at a protein concentration similar to ours, a value of 5.35 at 40 °C, leading to 5.66 at 20 °C corrected with a $\Delta H$ of 27 kJ/mol (Tanokura et al. 1978). Since the NMR studies are done in $D_2O$ and the ultrasonic investigations are carried out in $H_2O$, our result supports the assumption of Roberts et al. (1969) that isotope effects do not fundamentally influence the determination of pK values by NMR.

As shown in Fig. 3, a pronounced difference obviously exists between the ultrasonic titration curves of oxygenated and deoxygenated human hemoglobin A. The phosphate induced sound absorption of oxyhemoglobin has its maximum at around pH 6.75 compared to 6.85 in deoxyhemoglobin. Moreover, the maximal absorption of oxyhemoglobin solutions is 20% larger than that of deoxyhemoglobin. That means that the acoustic signal caused by buffer-protein proton-transfer in this case is an indicator for ligand-linked pK changes of the participating protein residues, namely α-amino and imidazole groups (Perutz 1970), which in the physiological pH range cause the alkaline Bohr effect.

As mentioned before, in human hemoglobin A there are 9 to 11 histidines and 2 α-amino groups per hemoglobin dimer, which are not masked and are probably free to interact with phosphate ions in the physiological pH range. Because an exact treatment of the hemoglobin-phosphate interactions would lead to a very complex system of coupled equations, a simplified approach was made by calculating averaged pK values for oxy and deoxyhemoglobin, assuming 13 identical groups per hemoglobin dimer. The results of the curve fitting procedure are given in Table 1. The quoted values are means and standard deviations of the best fits gained from measurements done at three different frequencies, 1.88, 5, and 18 MHz.

The optimized reaction volumes and reaction rate constants are in the same range as calculated for histidine and are very similar to the values obtained by Slutsky et al. (1984) from ultrasonic studies of interactions between inorganic phosphate and histidine residues of bovine pancreatic ribonuclease A. The average pK values reflect the ligand-linked pK changes, but are about 0.6 pH units lower than those determined by Brown and Campbell (1976) for hemoglobin with an NMR method. Since the actual pK spectrum may vary between 4.6 and 8.5 (Greenfield and Williams 1972; Matthew et al. 1980; Ohe and Kajita 1980; van Beek and de Bruin 1980; Russu et al. 1982) and also considerable variations of $k_f$ and $\Delta V$ are probable, here the simplified model leads only to qualitatively but not quantitatively satisfying results.

Concluding, we have shown that the additional sound absorption resulting from protein-phosphate interactions can be described theoretically by proton-transfer relaxation processes. The model can be used to calculate equilibrium parameters including pK values of titratable protein side chains from the measurement of the ultrasonic absorption as a function of pH. By choosing a proper pK value of the buffer, the pK range and therefore the type of side chain to be investigated can be selected. In proteins containing more than one titratable group, the determination of properties of single side chains would require additional blocking or masking procedures. Beside the fact that a relatively inexpensive experimental apparatus is needed for this kind of investigation, other advantages are the possibility of avoiding the use of any isotopes or labels and to simulate the physiological composition of the solvent. Our investigations also show that not all commonly used buffers are appropriate if the intrinsic sound absorption of proteins is to be measured, as they can cause acoustically relevant protein-buffer interactions.

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