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Proteins contribute insignificantly to the intrinsic buffering capacity of yeast cytoplasm

Jaroslaw Poznanski [a], Pawel Szczesny [a,b], Katarzyna Ruszczyńska [a], Piotr Zielenkiewicz [a,b], Leszek Paczek [c,*]

[a] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland
[b] Institute of Experimental Plant Biology and Biotechnology, Faculty of Biology, University of Warsaw, Warsaw, Poland
[c] Department of Immunology, Transplantology and Internal Medicine, Warsaw Medical University, Warsaw, Poland

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ABSTRACT

Intracellular pH is maintained by a combination of the passive buffering of cytoplasmic dissociable compounds and several active systems. Over the years, a large portion of and possibly most of the cell's intrinsic (i.e., passive non-bicarbonate) buffering effect was attributed to proteins, both in higher organisms and in yeast. This attribution was not surprising, given that the concentration of proteins with multiple protonable/deprotonable groups in the cell exceeds the concentration of free protons by a few orders of magnitude. Using data from both high-throughput experiments and in vitro laboratory experiments, we tested this concept. We assessed the buffering capacity of the yeast proteome using protein abundance data and compared it to our own titration of yeast cytoplasm. We showed that the protein contribution is less than 1% of the total intracellular buffering capacity. As confirmed with NMR measurements, inorganic phosphates play a crucial role in the process. These findings also shed a new light on the role of proteomes in maintaining intracellular pH. The contribution of proteins to the intrinsic buffering capacity is negligible, and proteins might act only as a recipient of signals for changes in pH.

1. Introduction

A wide range of physiological processes in living cells is dependent on the concentration of hydrogen ions, which influences metabolites, proteins and even cellular processes (reviewed in [1]). Additionally, the energy transduction machinery of cells runs mainly on proton-coupled transfer reactions and H^+ gradients. Therefore, regulation of intracellular pH (pH_i) is not just an element of maintaining cellular homeostasis but a central factor in the organisation of living systems. The most basic level of such regulation is the intrinsic buffering by weak acids and bases, such as ammonium, inorganic phosphates, amino- and carboxy- termini of proteins and protonation of the side chains of amino acid residues. These weak acids and bases constitute a complex buffer that passively reacts to localised changes in pH_i. The central system is a reversible interconversion of CO_2 and H_2O to HCO_3^- and H^+. In various organisms, this conversion is either more passive or more active and depends not only on the availability of additional proteins accelerating this process (such as carbonic anhydrases) but also on the efficiency of the respiratory system. The active maintenance of the concentration of hydrogen ions begins with proton translocating ATP-ases, such as yeast Pma1p, which is the master regulator of cellular pH [2]. Their activity is complemented by alkali cation/H^+ exchangers, such as the ones for Na^+/H^+ (NHEs); however, their role does not appear to be fully quantified yet [3]. Finally, compartmentalisation constitutes the last level of pH_i regulation, in which each cellular compartment often has its own specific pH, supplemented by intrinsic proton leakage [4]. The maintenance of organelle pH homeostasis is not just a local endeavour because pH influences cellular spatial dynamics via, for example, secretory pathways. Under stationary conditions, these systems working in concert are capable of maintaining the pH level with astonishing accuracy, reaching one tenth of a pH unit.

Relatively little attention is devoted to the very basic level of pH regulation, that is, intrinsic (non-carbonate) buffering by weak acids and bases. Because there are no other compounds with a pK_a close to physiological pH in cells at sufficiently high concentration, the intrinsic buffering capacity is typically defined by a sum of the contributions from phosphate groups and amino acid side chains [5]. In yeast, the cellular concentration of inorganic phosphate is estimated to be approximately 50 mM [6], and the pK_a of the $H_2PO_4^{2-}$ ion is reported to be in the range of 6.8–7.2 (depending on the conditions of the experimental determination of pK_a). This pK_a makes phosphates an excellent buffering candidate, and phosphates have indeed been directly implicated as a

* Corresponding author. Address: Department of Immunology, Transplantology and Internal Medicine, Warsaw Medical University, Ul. Nowogrodzka 59, 02-006 Warsaw, Poland.
E-mail address: leszek.paczek@wum.edu.pl (L. Paczek).

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prominent cellular buffer [7]. Despite that finding, proteins are still assumed to play a significant role in the passive buffering of hydrogen ions.

The buffering capacity of proteins comes from side chains of amino acids, amino and carboxyl ends of polypeptide chains and dissociable posttranslational modifications (PTMs). The exact nature of such contributions also depends on electrostatic interactions. However, the 3D structures or PTMs are not known for all proteins; therefore, rough approximations of isoelectric points, pK_a values or titration curves were predicted from protein sequences alone with satisfactory results [8]. The main assumption behind these calculations (that all charged amino acid residues are exposed to solvent) is not true for a large number of proteins [9]. However, the accurate predictions using this approximation result from the fact that, in an environment with physiological pH values, only histidines (and other compounds containing an imidazole group, for example, free L-histidine or histidine-containing dipeptides such as carnosine, anserine, and balenine) are capable of effectively capturing and releasing protons. This ability results from having pK_a values close to physiological pH (histidine has a pK_a of 6.04). The other candidate would be cysteine, with a pK_a value of 8.3; however, cysteine has complex biochemistry, such as the formation of disulphide bridges, involvement in regulation and oxidation sensing. Therefore, its contribution to pH buffering is relatively minor.

In this study, we assessed the intrinsic buffering capacity of proteins in yeast, based on detailed abundances of proteins in a whole cell. We calculated the intrinsic buffering capacity under the assumption that histidines are responsible for the majority of the buffering effect. Then, we compared this value to reported buffering capacities and to our own potentiometric titration of yeast cytoplasm. We show that there is a significant difference of almost three orders of magnitude between the total buffering capacity and the contribution to this capacity from proteins.

2. Materials and methods

2.1. Data sources

Protein abundance data for *Saccharomyces cerevisiae* were taken from a single-cell proteomic analysis [10]. The cell volume was reported from original sources on the basis of a search in the Bionumbers database.

2.2. Approximation of proteins' buffering effect

Typical pH values in mesophilic organisms vary between 6 and 9 (±1.5 units from pH of 7.5). Therefore, we assumed that in conditions outside that range, a cell either dies or some mechanisms that actively maintain acid–base homeostasis become involved. This assumption allows us to assess the buffering effect of proteins by only considering histidine side chains. We do not account for cysteine because of the presence of its reactive thiol group. The buffer concentration C_{buf} can be approximated (1) using the number of histidine side chains (N_{his}) per protein, protein abundance data (expressed in number of moles of a protein, N_{mol}) and the volume of the cell (V):

$$C_{buf} = \frac{\sum N_{his} N_{mol}}{N_A V} \quad (1)$$

We used the Uniprot sequence database to obtain the number of histidines per protein.

2.3. Calculation of buffering capacity and protonation level

Buffering capacity (β) can be defined as the quantity of strong acid or base that must be added to change the pH of one litre of solution by one pH unit:

$$\beta = \frac{dn}{d\mathrm{pH}} \quad (2)$$

In Eq. (2), n is the number of equivalents of strong base that was added (addition of dn moles of acid will change pH in the opposite direction). Assuming that the strong base is monoprotic and the total volume is one litre, we can treat the concentration and number of moles interchangeably. With this definition, the buffer capacity of a weak acid can be expressed as in Eq. (3):

$$\beta = 2.303\left(\frac{K_w}{[H^+]} + [H^+] + C_{buf}K_a\frac{[H^+]}{(K_a + [H^+])^2}\right) \quad (3)$$

where the buffer capacity depends on the concentrations of buffer (C_{buf}) and H^+ ($[H^+]$) and the acid dissociation constant K_a. K_w denotes the self-ionisation constant of water. We assumed that amino acid residues behave as independent monoprotic acids.

An explicit theoretical titration curve, used in the model fitted to the experimental data, was obtained by symbolic integration of Eq. (2) combined with Eq. (3), giving

$$n(\mathrm{pH}) = \left(10^{\mathrm{pH}-\mathrm{p}K_w} - 10^{-\mathrm{pH}} + \sum\frac{C_{buf}}{1+10^{\mathrm{p}K_a-\mathrm{pH}}}\right) - \left(10^{\mathrm{pH}_0-\mathrm{p}K_w} - 10^{-\mathrm{pH}_0} + \sum\frac{C_{buf}}{1+10^{\mathrm{p}K_a-\mathrm{pH}_0}}\right) \quad (4)$$

where the summation is over all titratable species and pH_0 is the initial pH of the sample. The model was fitted to the experimental data using the Marquardt–Levenberg algorithm [11] implemented in the Gnuplot program.

2.4. Degree of protonation

When pH = pK_a, the degree of protonation is 1/2. Assuming a deprotonation reaction (and one state transition), we can calculate the degree of protonation (Omega) using the following equation:

$$Omega(\mathrm{pH}) = \frac{1}{1+10^{(\mathrm{p}K_a-\mathrm{pH})}} \quad (5)$$

2.5. Potentiometric titration of yeast cytoplasm

Cells of the yeast strain W303 (*MAT*a {*leu2–3,112 trp1–1 can1–100 ura3–1 ade2–1 his3–11,15*}) transformed with the pRS426 plasmid were grown at 30 °C in one litre of standard synthetic complete liquid medium (SC-ura) with vigorous shaking until reaching an optical density (OD) of ~1.5, monitored at 600 nm. The cells were then centrifuged, washed twice with water and suspended in 12 mL of 20 mM Tris buffer, pH 7.5. Next, 12 mL of glass beads were added. The cell extracts were prepared by vigorously shaking the prepared mixture for 60 s followed by 60 s of chilling in ice, repeated ten times. After centrifugation at 8000 rpm for 15 min, the supernatant of the extract was divided into 2 mL samples and centrifuged twice at 14000 rpm for 15 min at 4 °C to remove membranes and organelle fractions. Finally, the remaining cytoplasm was stored in a deep freezer. Potentiometric titration was performed using 1 mL of freshly unfrozen cytoplasm sample. The initial pH of the cytoplasm sample was 6.02, and the titration was performed with the stepwise addition of 0.5 M NaOH and monitored with the aid of a Thermo Orion Star pH-meter equipped with an 8102NUWP glass electrode.

The titration model (see Eq. (4)) concerned two types of species, e.g., cytoplasm with unknown values of C_{buf} and pK_a and 20 mM TRIS with C_{buf} = 20 mM and pK_a = 8.10. The initial pH of the sample, pH_0, was set to 6.02.

2.6. Titration of yeast cytoplasm monitored using ^{31}P NMR spectroscopy

^{31}P NMR spectra of cytoplasm samples (selected from the large number of samples from potentiometric titration, see above) were collected at 161.897 MHz on a Varian Inova-400 MHz spectrometer equipped with a 5 mm inverse broadband pfg probe. All experiments were acquired at 25 °C using an s2pul pulse sequence with a spectral width of 6.5 kHz, 20802 points and 512 scans. The pH-induced changes in the location of individual resonance lines were analysed assuming a two-state protonation equilibrium based on the Henderson–Hasselbach formulae [12] according to the following equation.

$$\delta(\mathrm{pH}) = \delta_0 + \Delta\delta \cdot Omega(\mathrm{pH}) \quad (6)$$

where δ_0 is the chemical shift of the ^{31}P resonance signal for the protonated species, δ(pH) represents the experimental pH-dependent location of that signal in the ^{31}P NMR spectrum, and $\Delta\delta$ is the estimated chemical shift change upon proton dissociation. The function Omega(pH) (see Eq. (5)) describes the titration of a given species. The resonance signal of the α phosphate group of ATP located at −9 ppm was used as the internal reference because its location has been proven to not significantly vary in the analysed pH range of 6–9 [13].

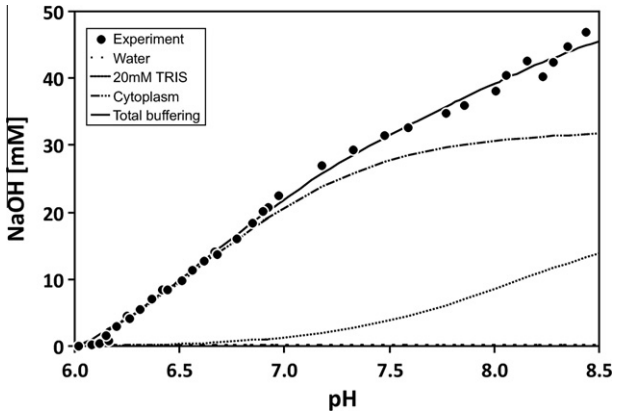

Fig. 1. Experimental titration data collected for yeast cytoplasm pretreated with 20 mM TRIS and its decomposition into two titratable components. The contribution from water dissociation is negligible.

3. Results

3.1. Assessment of proteins' contribution to total buffering capacity

A total of 19733072 histidine side chains in a volume of 37 μm^3 correspond to a concentration of 0.9 mM. As a result, the buffering capacity of proteins is estimated to be 0.1 mM at pH 7.2. The reported values of the total buffering capacity for yeast in the literature differed by an order of magnitude; therefore, we performed a

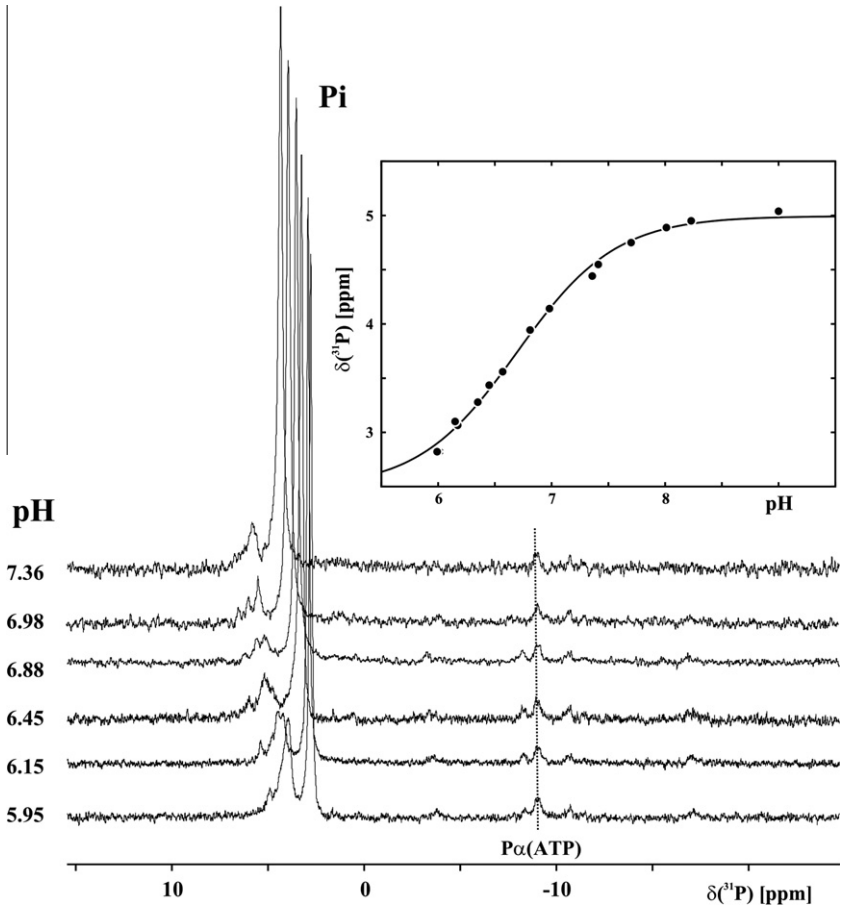

Fig. 2. pH-related changes in ^{31}P NMR spectra recorded for the yeast cytoplasm sample. In the inset, the titration curve is fitted to the changes in the location of the inorganic phosphate signal (P_i).

potentiometric titration of yeast cytoplasm. The titration curve fitted to the experimental data is shown in Fig. 1. The pK_a of the cytoplasm has been estimated to be 6.59+/−0.03, whereas its total intrinsic buffering capacity was estimated to be 41 mM+/−2 mM. This value should be attributed to the contribution of inorganic phosphate, which has a virtually identical pK_a value (6.70+/−0.06). This value was estimated independently using the ^{31}P NMR-monitored titration of the same cytoplasm sample (see Fig. 2), however it should be noted that other phosphorylated compounds may also contribute to buffering (see small signals left to P_i).

3.2. Calculation of degree of protonation

In addition to the buffering capacity, we assessed the degree of protonation of histidine side chains in soluble proteins. This parameter is only a function of pK_a and pH (see Eq. (5)). Therefore, we can easily calculate that at pH values of 7.1, 7.2 and 7.3, the average percentage of protonated histidines will be 7.3%, 5.9% and 4.7%, respectively. For the yeast proteome, this corresponds to roughly 5×10^5 histidine residues (2.6%) changing protonation state in a cell with a pH between 7.1 and 7.3.

4. Discussion

In this study, we have attempted to estimate the buffering capacity of proteomes in yeast. This estimate is roughly three orders of magnitude smaller than our measurement of the total intrinsic buffering capacity. Even considering the various approximations used, it is clear that in yeast and presumably in many other cells, rapid swings of pH are buffered by compounds other than proteins, such as inorganic phosphates or hydrated carbon dioxide. Charged side chains of amino acids only play a minor role in this process, as their overall concentration is rather small in comparison to the concentration of other buffering agents. For example, concentration of histidine residues changing their protonation state between pH of 7.1 and 7.3 is ca. 23 μM (2.6% of 0.9 mM). Both the theoretical assessment of the capacity of proteins to bind H^+ and the experimental tests do not support the simplified notion of the intracellular buffering mechanism. Given that relatively large differences of degrees of protonation are results of small changes in pH, it is clear that in the majority of cells, the proteome's role might not be direct buffering but most likely absorbing and reacting to signals of changes in pH.

The major findings of this study have significant implications. Non-bicarbonate buffering components have been shown to mediate most (~70%) of the spatial (localised) regulation of pH. However, this effect declined sharply at low pH_i [14]. Depending on the main intrinsic buffering agent (inorganic or protein-based), intracellular acidosis may lead to different outcomes. For inorganic compounds, the buffering capacity will simply decline with a large decline of pH_i. For proteins, low pH could lead to loss of activity, reduction of disulphide bonds or even degradation of proteins. The last element could most likely increase the total concentration of buffering agents in the cytoplasm and therefore could provide an additional mechanism that would reduce the negative effect of extreme intracellular acidosis.

5. Author contributions

Designing experiments: LP, PZ, JP. Theoretical calculations: PS. Experimental titration: JP. NMR measurements KR. Writing paper: PS, JP, KR, PZ, LP.

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