

## Reply to the letter

### DISTRIBUTIONS OF pI VERSUS pH PROVIDE PRIOR INFORMATION FOR THE DESIGN OF CRYSTALLIZATION SCREENING EXPERIMENTS: RESPONSE TO COMMENT ON 'PROTEIN ISOELECTRIC POINT AS A PREDICTOR FOR INCREASED CRYSTALLIZATION SCREENING EFFICIENCY'

In a recently published advanced access article, we described the use of a relationship between the distribution of pH of crystallization and the pI of the protein being crystallized to design more efficient crystallization screening experiments. The validity of our analysis has been questioned by Huber and Kobe. We wish to address their concern regarding our interpretation and clarify the electronic version of our article.

The motivation for our analysis is that although a number of studies have attempted to provide improved crystallization strategies (Gilliland *et al.*, 2002), much of the 'knowledge' disseminated about protein crystallization continues to be anecdotal, with little statistical evidence or control experiments to prove general efficiency or usefulness. Considering the large number of physical, chemical and method related parameters, very few are sampled (and reported) with sufficient overlap to allow their direct use as a predictor for optimizing crystallization success (Rupp, 2003). One parameter that is however frequently reported—regardless of the crystallization strategy employed—is the pH of the crystallization cocktail. Use of pH as a predictor for crystallization success, either globally or in correlation with the minimum solubility of a given protein at its isoelectric point, pI, appears attractive. Unfortunately, no direct correlation between minimum solubility at the pI and the pH of crystallization has ever been established (Samsudzi and Fivash, 1992; Farr *et al.*, 1998; Beretta *et al.*, 2000). However, a significant relationship in fact exists between the calculated pI of successfully crystallized proteins with  $pI < 7$ , and the reported pH at which they were most frequently crystallized, as we describe shortly.

Because pH is one of the few consistently reported parameters in the Protein Data Bank (PDB), we used the 9596 SEQRES records of a non-redundant protein dataset (excluding proteins/nucleic acid complexes) (Kantardjieff and Rupp, 2003), which contain the entire expressed construct sequence including any tags, fusions or linkers, to calculate the pI using the  $pK_a$  values of Bjellqvist *et al.* (1993). The frequency distribution for pI of proteins is bimodal (Fig. 1A), with highest frequencies (modes) at approximately pH 5.7 and 9.0, corresponding to the pI distribution for proteins encoded by sequenced genomes (e.g. see Urquhart *et al.*, 1998; Baisnee *et al.*, 2001; Adams *et al.*, 2003). The frequency distribution for reported crystallization pH of proteins is unimodal, with mean = 6.7, median = 6.9 and mode = 7.5 (Fig. 1B).

A similar crystallization pH distribution has been observed from unbiased random screening experiments in structural genomics initiatives (Hosfield *et al.*, 2003; Rupp, 2003; Rupp and Wang, 2004).

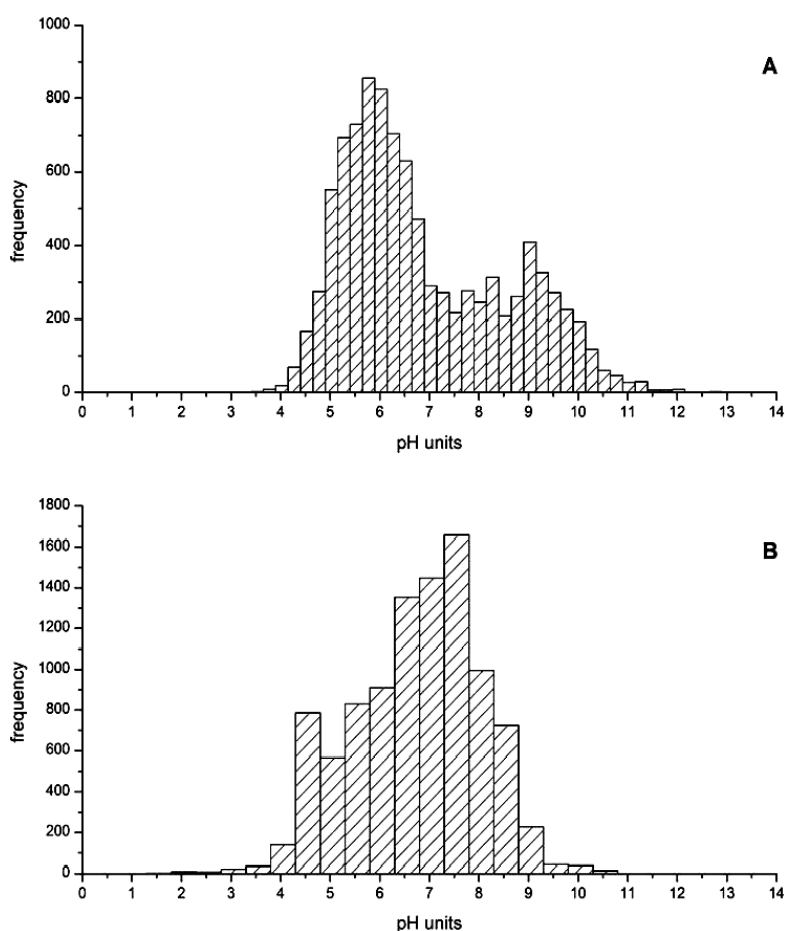
In our original article, we reported that empirical distributions of the observed data imply a preferred range of crystallization pH for acidic and basic proteins, and these preferences provide strong prior information for the design of crystallization screening experiments of significantly increased efficiency. We thank Huber and Kobe for correctly pointing out that the correlation line depicted in Figure 2 of our original article is a result of our transformation of the data. Indeed, this confusing presentation has already been brought to our attention (P.S. Stewart, personal communication). However, the correlation reported erroneously in that figure was never used to produce a predictive model via regression analysis, which Huber and Kobe overlooked.

Transformation of the data preserves the experimental connection between a pI and its corresponding crystallization pH. In practice, the distributions of (pI versus pH) and (pI versus pI–pH) are identical, as verified by quantile–quantile plots. Huber and Kobe's distributions are not equivalent to ours, because the empirically observed pH and pI data (Fig. 1) are clearly not uniformly distributed and do contain information. In fact, one observes statistically significant non-zero correlations between pH and pI. Clustering of the bivariate observed data pI and pH, using the posterior probabilities from a mixture of Gaussian distributions, led us to assess the data in two separate groups,  $pI < 7$  (6266 cases) and  $pI \geq 7$  (3330 cases). For each group, and for all the data together, we computed 99% confidence intervals for the Pearson correlation coefficient between pI and pH. These were (0.04, 0.10), (–0.08, 0.006) and (–0.003, 0.0496),<sup>1</sup> respectively, indicating that the correlation between pI and pH for the first group is significantly different from zero.

This statistical significance of the correlation allows a linear regression of pH on pI for the first group. SPSS was used to fit the linear regression model leading to  $pH = 5.8800.142 \times pI$ . The standard errors of the intercept and the slope are 0.137 and 0.023 respectively, with both estimates being significantly different from zero ( $P$ -values  $< 0.0001$ ). Based on the statistics related to this fit, a  $100(1 - \alpha)\%$  confidence interval for the expected pH levels can be obtained using

$$5.88 + (0.142 \times pI) \\ \pm z_{\alpha/2} \sqrt{1.536/6266 + pI - 5.82)^2 \times (0.023)^2}$$

<sup>1</sup>The confidence intervals were computed based on the distribution of the Fisher transformation of the Pearson correlation. We also computed these intervals using the bootstrap method, and the resulting intervals were in agreement.



**Fig. 1.** Frequency distributions for empirically observed PDB pH and pI data. (A) pI of successfully crystallized proteins. (B) Reported pH of crystallization for proteins.

where  $z_{\alpha/2}$  is the  $1 - \alpha/2$  quantile of the standard normal distribution. Common values of  $z_{\alpha/2}$  for 90, 95 and 99% confidence are 1.64, 1.96 and 2.57, respectively. This, for example, predicts that in repeated experiments with  $pI = 5$  the mean of the expected crystallization pH values will be between 6.54 and 6.64 with 95% confidence. The corresponding prediction interval, indicating the predicted pH value for a given pI in a single experiment, can be obtained using

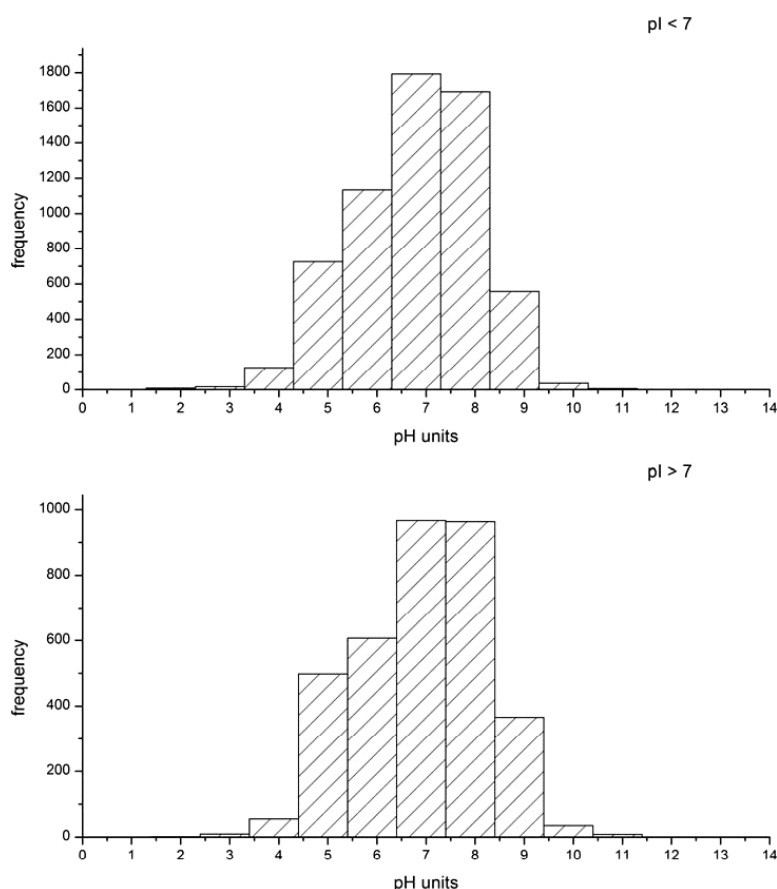
$$5.88 + (0.142 \times pI) \pm z_{\alpha/2} \sqrt{1.536 + 1.536/6266 + (pI - 5.82)^2 \times (0.023)^2}.$$

For  $pI = 5$ , the 95% prediction interval is (4.2, 9.0), indicating that in ~95% of the successful experiments the pH value will be in the range 4.2–9.0. The 50% prediction interval for  $pI = 5$  is narrower (5.8–7.4). Based on the fact that the correlation between pH and pI is not significant at the 1% level for cases other than the acidic proteins, we did not implement a predictive correlation model, but decided to follow the corresponding empirical distributions

of pI versus pH (equivalent to pI versus  $pI - pH$ ) shown in Figure 2.

CrysPred (<http://www-structure.llnl.gov/cryspred/>), the simple prototype pH range calculator described in our original article, shows how empirical distributions of observed crystallization pH for a given pI range can be used as prior information to optimize the efficiency of initial crystallization screening in HTPX by identifying with the highest overall efficiency (least material, supplies and resources, and thus cost) the proteins that are most likely to yield useful or suitable crystals and structures. The results can be easily imported into any customizable screen generator that allows to define the frequency of occurrence for selected pH ranges [e.g. CrysTool (Segelke and Rupp, 1998; Segelke, 2001)]. Supplemental information on the website discusses additional caveats regarding accuracy of pI calculations and reported pH data, and usage bias of the experimental frequency distributions (Rupp and Wang, 2004).

Crystallization is a special case of phase separation from a thermodynamically metastable solution under kinetic control (Rupp, 2003). As discussed in our original article,



**Fig. 2.** Frequency distribution of pH versus pI for successfully crystallized proteins. Top panel shows the frequency distribution of crystallization pH and calculated pI of successfully crystallized acidic proteins. Bottom panel shows this frequency distribution for basic proteins. Based on these distributions of empirically observed data, acidic proteins tend to crystallize 0–2.5 pH units above their pI, whereas basic proteins prefer to crystallize 0.5–3 pH units below their pI.

while control over kinetic parameters such as nucleation or growth rates is rather difficult to achieve, attractive interaction between molecules as a thermodynamically necessary—but not sufficient—condition for crystallization can be described on the basis of thermodynamic excess properties, such as their manifestation in the second virial coefficient,  $B_{22}$ . pH and electrolytes have been shown to be crucial solvent parameters that modulate potentials through specific and non-specific effects (Benas *et al.*, 2002; Retailleau *et al.*, 2002). Because charge distributions on proteins are discrete, and distances between charged residues on a protein surface are not negligible compared to the protein diameter, the specificity of charge interactions cannot be ignored, and colloidal model systems (Belloni, 2000; Frenkel, 2002) have not been successful in describing these interactions in proteins (Piazza, 2000; Tardieu *et al.*, 2002; Allahyarov *et al.*, 2003).

While methods for choosing protein crystallization conditions have largely been empirical (McPherson, 1982), with the recent development of random screening methods (Segelke and Rupp, 1998; Segelke, 2001), it is anticipated that statistical

analysis will provide predictive frameworks that increase the probability of producing high-quality crystals (Rupp and Wang, 2004). Empirical distributions of observed PDB data for acidic and basic proteins, as well as for smaller groups of data binned by pI, suggest that acidic proteins crystallize with highest likelihood  $\sim$ 0–2.5 pH units above their isoelectric point, whereas basic proteins preferably crystallize  $\sim$ 0.5–3 pH units below their isoelectric point (with the sum of these related distributions adding up to the distribution shown in Fig. 1). This conclusion is based on the experimental facts (the pI versus pH distribution of observed data), and Huber and Kobe's statement 'We have demonstrated here that this conclusion is based on misinterpretation of data and should not be used to guide crystallization experiments until a correlation between pH and pI is established' is untenable. Following an experimental distribution suitable for the selected pI range to increase overall efficiency is completely valid. Furthermore, for the pI < 7 group, a correlation with crystallization pH has been established, which could be used to support a predictive model. The absence of negatives in the PDB data does not

allow the calculation of actual propensities, however, and it is likely that experimentally observed distributions are biased by usage (Rupp and Wang, 2004). Thus, a predictive model based on correlations would be no more reliable at this point than following the empirical distributions of pH for a given range of pI. Our statistical analysis suggests favorable pH distributions and ranges for improved efficiency of crystallization screening experiments, validating early grid designs that varied pH around physiological values (McPherson, 1982).

## DISCLAIMER

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