

## SUPPLEMENTARY MATERIAL

### **Dissecting electrostatic interactions in *Bacillus circulans* xylanase through NMR-monitored pH titrations**

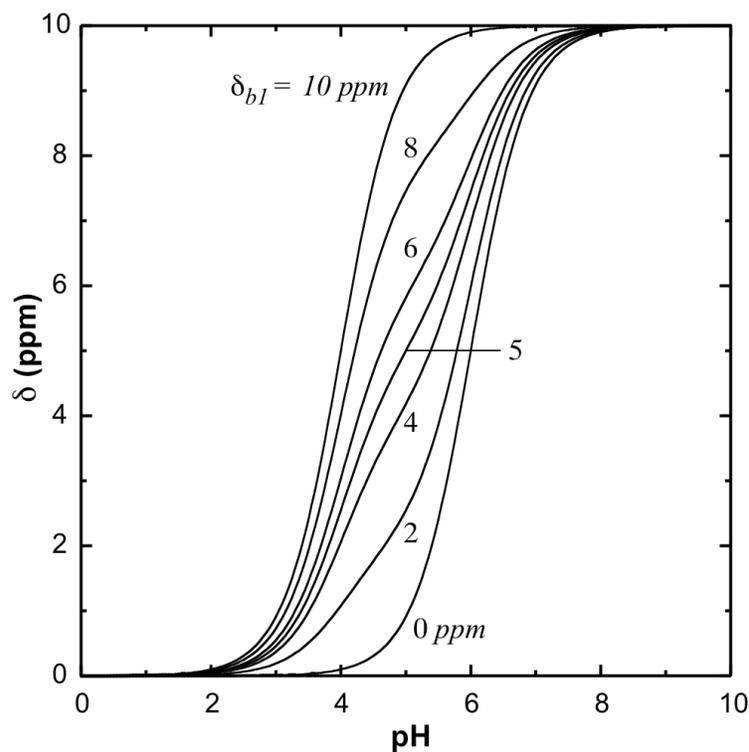
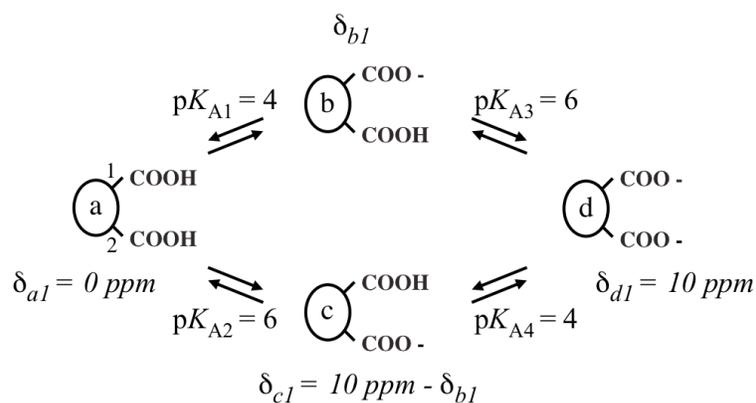
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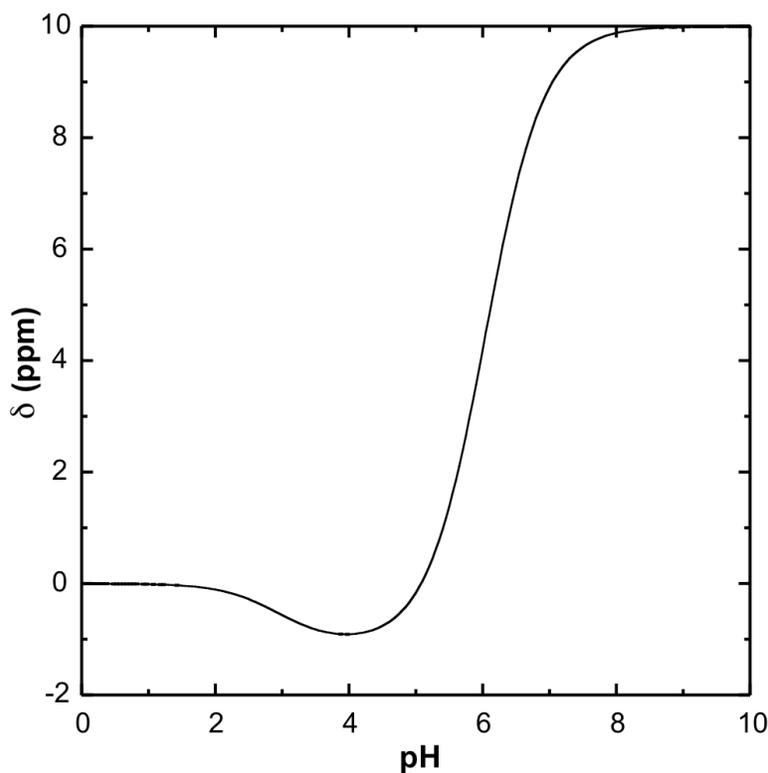
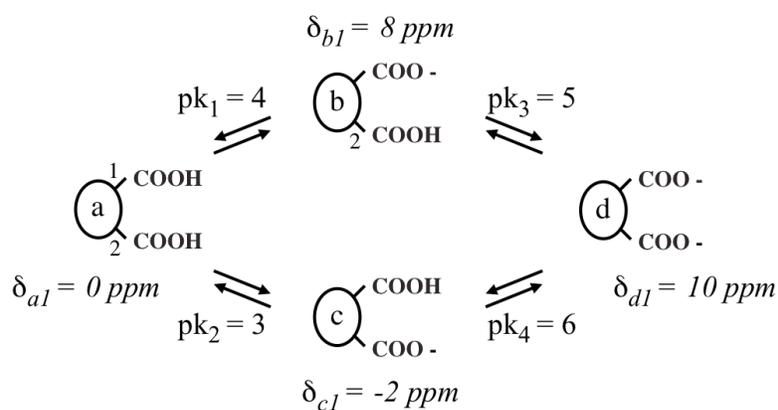


**Supplemental Figure S1.** Simulated chemical shift pH-dependence of a reporter nucleus in one of two carboxylic acids following case 3 (thermodynamically independent equilibria, but with chemical shifts dependent additively on the ionization state of both residues). Error-free data were generated using equation (7) with  $\text{p}K_{A1} = \text{p}K_{A4} = 4$  ( $\alpha = 1$ ), and total change in chemical shift ( $\delta_{d1} - \delta_{a1}$ ) of 10 ppm for site 1, along with  $\text{p}K_{A2} = \text{p}K_{A3} = 6$  for site 2. The intermediate changes in chemical shift are 10, 8, 6, 5, 4, 2, or 0 ppm due to the ionization of site 1 itself ( $\delta_{b1} -$

$\delta_{a1} = \delta_{d1} - \delta_{c1}$ ) and 0, 2, 4, 5, 6, 8, or 10 ppm due to the ionization of site 2 ( $\delta_{c1} - \delta_{a1} = \delta_{d1} - \delta_{b1}$ ). Although only applicable to the outer most (monophasic) curves, non-linear least squares fitting to a single Henderson-Hasselbalch titration (equation 4) yields  $pK_A$  values of 4.000, 4.256, 4.691, 5.000, 5.309, 5.744, and 6.000, respectively. In all cases, the true  $pK_{A1}$  of site 1 is 4, while the fit  $pK_A$  values progressively shift towards  $pK_{A2} = 6$  as the perturbation in chemical shift due to the ionization of site 2 becomes increasingly dominant. Use of the modified Hill equation (14) yields similar  $pK_A$  values, and  $n$  ranging from 0.5 (for the middle curve) to 1.0 (outer most curves). This implies varying degrees of negative cooperativity, despite using  $\alpha = 1$  to simulate the data.

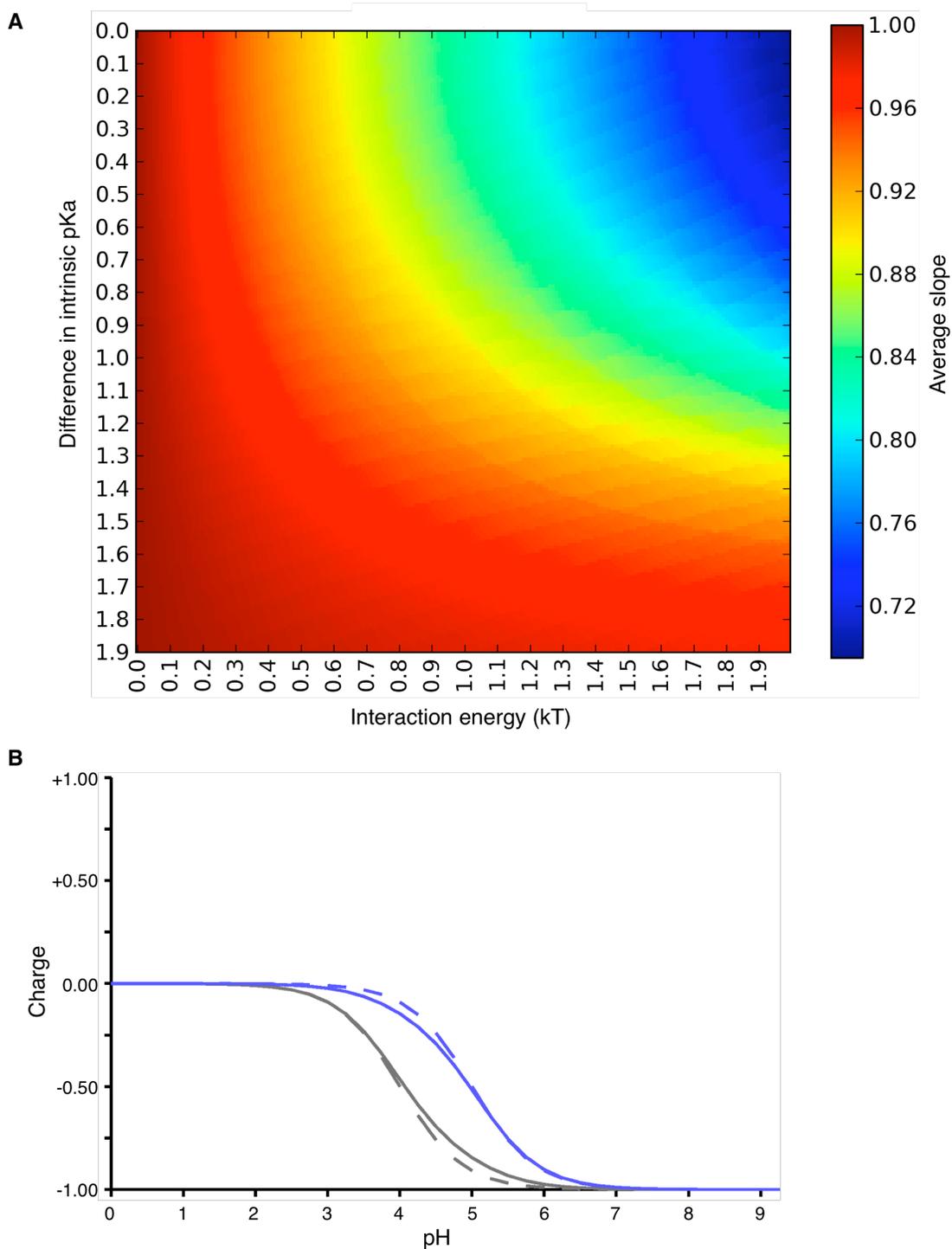
Fitting of the 5 inner most curves to equation (11a) yields macroscopic values of  $pK_{AI} = 3.996$  and  $pK_{AII} = 6.004$  and intermediate shifts  $\delta_{i1}$  of 7.941, 5.980, 5.000, 4.020, and 2.059 ppm (left-to-right), matching exactly those expected from the defining relationships (equations 9 and 12). Thus, in each case, accurate macroscopic  $pK_A$  values are extracted from the data, although the interpretation of these values in terms of the microscopic ionization behaviour of sites 1 and 2 and of the resulting chemical shift changes requires additional information.

Fitting of the five inner most curves to equation (5a) for case 2 yields apparent microscopic  $pK$  values of ( $pK_{A1} = \underline{4.096}$ ,  $pK_{A2} = 4.682$ ,  $pK_{A3} = \underline{5.904}$ , and hence  $pK_{A4} = 5.318$ ), (4.219, 4.392, 5.781, 5.609), (4.297, 4.297, 5.703, 5.703), (4.392, 4.219, 5.609, 5.781), and (4.682, 4.096, 5.318, 5.904), respectively. In all cases, these microscopic  $K_{Ai}$  values combine to give the expected  $pK_{AI}$ ,  $pK_{AII}$  and  $\delta_{i1}$  values (equations 6 and 9). The underlined numbers correspond to the apparent major ionization pathway with increasing pH and thus approximate the input  $pK_{Ai}$  values of 4 for site 1 and 6 for site 2. Although the fitting is precise, the assumption that chemical shift changes arise only from the ionization of site 1 leads to the incorrect conclusion of a branched equilibrium with  $\alpha$  values ranging from 0.039 to 0.060.



**Supplemental Figure S2.** Simulated chemical shift pH-dependence of a reporter nucleus in one of two carboxylic acids following a general example of coupled or branched equilibria with chemical shifts dependent upon the ionization of both residues in a non-additive manner. The curve was generated using equation (3b) with  $pK_{A1} = 4$ ,  $pK_{A4} = 6$  (thus  $\alpha = 0.01$ ),  $\delta_{a1} = 0 \text{ ppm}$ ,  $\delta_{b1} = 8 \text{ ppm}$ ,  $\delta_{c1} = -2 \text{ ppm}$ , and  $\delta_{d1} = 10 \text{ ppm}$  for site 1 and  $pK_{A2} = 3$  and  $pK_{A3} = 5$  for site 2. However, these values cannot be recovered uniquely by non-linear least squares fitting to this underdetermined equation. Fitting to equation (4) for a single Henderson-Hasselbalch titration

gives a  $pK_A$  value of 6.12. Although obviously unsatisfactory, this is reasonably close to  $pK_{A4} = 6$  for site 1. Fitting to equation (11a) yields robustly the macroscopic values of  $pK_{AI} = 2.959$ ,  $pK_{AII} = 6.041$ , and  $\delta_{a1} = 0$  ppm,  $\delta_{i1} = -1.091$  ppm, and  $\delta_{d1} = 10$  ppm, as expected from the defining equations (9) and (12). Note that in this situation, site 2 ionizes predominantly before site 1 with increasing pH, and thus  $pK_{AI} \sim pK_{A2}$  and  $pK_{AII} \sim pK_{A4}$ . In other words, the macroscopic  $pK_{AI}$  and  $pK_{AII}$  approximate closely the microscopic  $pK_{Ai}$  values of the sites 2 and 1, respectively, along the lower branch of the drawn pathway. These data cannot be fit adequately to equation (5a) for case 2, as in this specific model, chemical shift changes are only due to the ionization of site 1 itself and thus must occur in a uniform direction. Fitting to a modified Hill equation (14) yields  $pK_{A'} = 6.11$  and  $n=1.16$ , suggesting positive cooperativity despite being simulated with a negative interaction factor of  $\alpha = 0.01$ .



**Supplemental Figure S3.** (A) The titration behavior of a coupled two carboxylic acid system plotted as a function of the difference in intrinsic  $pK_{Ai}$  values (i.e.  $|pK_{A1} - pK_{A2}|$  from Figure 1) and the electrostatic interaction energy ( $E_{\text{int}} = 2.303\Delta pK_A$ ). The color scale reports the average slope of fitting the two titration curves to the linearized form of the Henderson-Hasselbalch

equation. A slope of 1.00 indicates perfect monophasic Henderson-Hasselbalch behavior, whereas lower values indicate increasing deviations from ideal sigmoid titration curves. Biphasic coupled titrations are observed when a significant electrostatic interaction is present and when the  $pK_{A1}$  and  $pK_{A2}$  values are within  $\sim 1.3$  units of each other. Otherwise, the titrations occur independently or sequentially (i.e. one branch of the scheme shown in Figure 1 dominates) with near monophasic behavior, respectively. This is exemplified in (B) by comparing the titration curves for an uncoupled system ( $E_{int} = 0.0$ ) with  $pK_{A1}$  and  $pK_{A2}$  differing by 1 (dashed lines), and a coupled system ( $E_{int}=1.0kT$ ) with a  $pK_{Ai}$  difference of 0.6 (solid lines). Whereas the uncoupled system has the expected average slope of 1.0, the coupled system visibly deviates from Henderson-Hasselbalch behavior with an average slope of 0.86. Note that such differences would not appear if the titration curves were measured coarsely at pH intervals larger than  $\sim 0.5$  units. Figures produced with pKaTool (Nielsen JE (2007) *J Mol Graph Model*, 25:691-699).