ABSTRACT

Observing and measuring charge regulation between two redox states of myoglobin using protein charge ladders and capillary electrophoresis

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Charge regulation occurs when a protein modulates its net charge in a way that minimizes some perturbance, e.g. the binding of a metal cofactor. Net charge is a fundamental biophysical property that is nonetheless poorly characterized for many proteins. Although charge regulation has been observed in a handful of cases, the extent to which this phenomenon occurs, its mechanisms, and its function are not well understood (1,3). This study examined charge regulation in myoglobin in the transition between two redox states of the heme iron, oxy-Mb (Fe^{2+}) and met-Mb (Fe^{3+}). Protein charge ladders and capillary electrophoresis were used to measure the net charge of each myoglobin species ($Z_{oxy\text{-}Mb}$ and $Z_{met\text{-}Mb}$). It was determined that $Z_{oxy\text{-}Mb} = -1.67 \pm 0.09$, while $Z_{met\text{-}Mb} = -1.02 \pm 0.06$. The difference between the net charge of these two states, 0.6 ± 0.1, was smaller than the expected 1.0 unit. The net charge of myoglobin was regulated by 0.4 ± 0.1 units in the transition between oxy-Mb and met-Mb. This study demonstrated the existence of charge regulation in myoglobin, suggesting that this phenomenon may be more widespread than previously considered.

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OBSERVING AND MEASURING CHARGE REGULATION BETWEEN TWO REDOX STATES OF MYOGLOBIN USING PROTEIN CHARGE LADDERS AND CAPILLARY ELECTROPHORESIS

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By

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CHAPTER ONE

Introduction

Protein Net Charge: a Relevant Biophysical Property

The net charge of a protein is a relevant but often overlooked biophysical property that can have significant implications for a protein's function (1). Protein charge arises from the contributions of ionizable amino acid residues as well as any tightly bound charged cofactors (protons, metal ions) (1). Localized charge distributions can vary significantly throughout the protein and are often physiologically relevant; for example, Fried et. al. recently demonstrated the catalytic function of a localized electric field within the active site of ketosteroid isomerase (2). These local fluctuations are more often considered and generally better-understood than net charge (1).

A protein's net charge constitutes a so-called "bird's eye view" of its electrostatic surface potentials and can affect its interactions with other charged species (1,3). At physiological pH this includes a significant proportion of biologically relevant molecules, including many carbohydrates, phospholipids, and typical cofactors as well as nearly all proteins (3). For example, net charge can have a significant impact on aggregation, a hallmark of neurodegenerative diseases. In fact, a recent study demonstrated that in human superoxide dismutase-1 (SOD1), changes to the protein's net charge decreased its aggregation potential (4). Thus, understanding protein net charge and the myriad factors that affect it could have enormous potential for understanding the physiology and pathology of a variety of biological processes.

It has traditionally been assumed that the net charge of a protein can be determined from its amino acid sequence and textbook pK_a values (1). The first step in determining the net charge of a given protein using this method requires using its amino acid sequence to find all of the ionizable residues (those capable of associating/dissociating a proton) (5). The ionization state of each residue can then be predicted using its textbook pK_a value, the pH of the relevant solution, and the Henderson-Hasselbalch equation (5). The charge values for each ionizable residue (determined using the aforementioned method) are then simply summed to give the protein's net charge (5).

This method is simple and convenient, and has traditionally been used with little challenge to predict the net charge of proteins in solution. However, the values that this method predicts are often inaccurate in practice, due largely to the significant degree of variability in the pK_a values of the ionizable residues (1,3,6). It has been experimentally shown that sidechain pK_a values can differ by as many as five units from textbook values. This led to the development of analytical techniques to experimentally measure protein net charge. Several experimental strategies exist, including isoelectronic point (pI) determination; however, these strategies are not ideal, exhibiting limited accuracy across a variety of conditions (pH, ion concentration) and/or requiring large amount of protein (1,7). Protein charge ladders have emerged as a convenient and accurate analytical tool for experimentally measuring protein net charge.

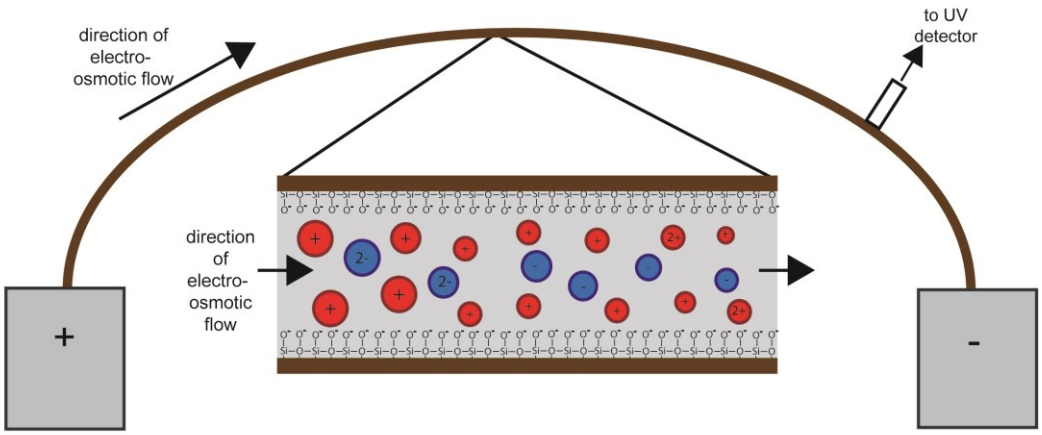


Figure 1: Cartoon schematic of capillary electrophoresis. The bulk solution moves through the capillary by interacting with the fused silica walls and migrating towards the negative electrode under the applied voltage. Sample molecules move based on their resistance to the bulk flow (hydrodynamic drag) and net charge.

Measuring Net Charge Using Protein Charge Ladders

Protein charge ladders are a relatively simple analytical tool that enables experimental quantification of a protein's net charge. A protein charge ladder is constructed by reacting a protein with a given number of molar equivalents of an acetylating agent under basic conditions (1,3,6,7). This reaction causes several of the lysine residues (ε-NH_3^+), which are protonated at physiological pH, to become acetylated (ε-$NHCOCH_3$); this effectively eliminates 0.9 units of positive charge from the total charge of the protein (1,8). (Although the expected change would be a single integer unit of charge, it has been demonstrated that the actual change is 0.9 units (8). For more information see the section entitled "Charge Regulation" below.) It is impossible to control the exact number of lysine residues that are acetylated on each protein in the solution, so the result of this reaction is a mixture of acetylated derivatives of the original

protein (1,3,6,7). These derivatives differ by integer numbers of acetylations, which is to say multiples of 0.9 units of net charge. This mixture of derivatives can be easily separated using capillary electrophoresis (1,3,6,7).

Capillary electrophoresis (CE) separates species on the basis of two factors: net charge and hydrodynamic drag (1,3,6,7). Figure 1 depicts a simplified schematic of this process. The electrophoretic mobility of a species (μ) is directly proportional to its net charge (q) and inversely proportional to its hydrodynamic drag, represented by the coefficient of friction (f_{eff}):

$$\mu = \frac{q}{f_{eff}} = \frac{eZ}{f_{eff}} \qquad (1) \qquad \text{Eq. 1}$$

For a particular species, its charge in coulombs can be represented by eZ, where e is the charge of an electron and Z is the charge of the species in unit multiples of e.

Because of the relatively small size of the acetyl group compared to a protein, acetylation typically has a negligible effect of a protein's hydrodynamic drag (which is a product of size and shape). Thus, the only factor that affects the separation of the acetylated derivatives comprising a protein charge ladder is their charge (1,3,6,7). The result of this separation is an electropherogram in which each peak represents a different acetylated derivative. The different derivatives, or "rungs," thus constitute a self-calibrating ladder that can be used to extrapolate the net charge of the unmodified protein (1,3,6,7). This technique has been used to quantify the net charge of a variety of biologically-relevant proteins. It has also been used to explore protein net charge as a fundamental biophysical property, for example revealing phenomena such as charge regulation (1,3,6,7,8).

Charge Regulation

Protein charge ladders have enabled the detection and exploration of charge regulation, a phenomenon whereby a protein modulates its overall net charge upon some disruption (e.g. acetylation or the binding of a charged cofactor) in a way that minimizes the change in net charge (1,3,8). Charge regulation occurs because a protein's ionizable amino acid residues exist in a nonlinear network in which changes to the protonation state of one residue can affect the pK_a (and thus the ionization state) of other residues in the network in ways that are complex and difficult to predict (1,3).

Menon et. al. observed this phenomenon in a wide variety of proteins upon lysine residue acetylation. They demonstrated that the change in net charge upon acetylation (ΔZ_{ac}) is less than the expected 1.0 unit (8). In other words, when the charge of a lysine residue is eliminated, the ionizable network of charges in the entire protein is affected in a way that slightly reduces the overall change in net charge (8). The Whitesides lab subsequently calculated that $\Delta Z_{ac} \approx 0.9 \pm 0.05$ for most proteins at typical physiological pH values and ionic concentrations (although this linear relationship begins to break down after ~5 acetylations) (1).

Charge regulation has also been observed upon metal binding in human superoxide dismutase 1 (SOD1), an important and widespread enzyme that scavenges oxygen free radicals and has also been implicated in the neurodegenerative disease amyotrophic lateral sclerosis (ALS) (3). SOD1 typically exists as a homodimer in which each subunit binds one copper(II) and one zinc(II) for a total of four divalent metal cations. The predicted change in net charge when converting between the demetalated state, apo-SOD1, and the typical metalated state, holo-SOD1, is therefore 8 units.

However, Shi et. al. found that the net negative charge of SOD1 actually decreased by approximately five units rather than the expected eight (3). Upon a significant disruption (i.e. the binding of four metal cofactors) SOD1 regulated its net charge in a way that decreased the overall change. Shi et. al. hypothesized that the large net negative charge of SOD1 plays some key biological role, thus requiring charge regulation to maintain the net negative charge even with the binding of essential metal ion cofactors (3). The extent to which charge regulation occurs within metalloproteins is currently unknown.

Metal Binding

It is estimated that one third of all known proteins, and one half of all known enzymes, coordinate at least one metal ion (9). These metal cofactors serve a variety of functions. Metals can aid in catalysis, for example by stabilizing a charged transition state in an enzyme's active site; metals can also serve structural roles by stabilizing a protein's conformation, as with zinc fingers or the milk protein α-lactalbumin (5,9,10). Some proteins bind metals as a means of sequestering them or storing them for later use, as with ferritin (5). Metals can also help coordinate other cofactors for transport or storage (5). Two of the most well-known metalloproteins, hemoglobin and myoglobin, contain a heme iron that coordinates oxygen for these two purposes (respectively) (5, 11).

The impact of metal binding upon net charge has not been studied for most metalloproteins and is not currently well understood. However, the Shi et. al. study regarding charge regulation in SOD1 indicates that, at least for some proteins, the relationship between metal binding and net charge is more complicated than simply adding the net charge of the apo-protein and the formal charge of the metal cofactor (3). A more complete understanding of effect that metal binding has on a protein's net charge

could yield valuable information as to the prevalence and mechanism of charge regulation and provide insight into the role of net charge in the function of metalloproteins. This study addressed this deficit by examining the impact of iron on the net charge of myoglobin.

Myoglobin

Myoglobin was selected for this investigation primarily because it is a fully-characterized, well understood, and relatively inexpensive metalloprotein with readily available structural data and a wealth of information on its properties and biological function (11,12). Myoglobin (Mb) is a relatively small globular protein with a molecular weight of ~17 kDa. It is comprised of eight alpha helices (labeled A-H) joined by coil regions; this polypeptide envelops and, via its His64 and His93 residues, coordinates a heme: iron cation complex (5, 11) (Figure 2).

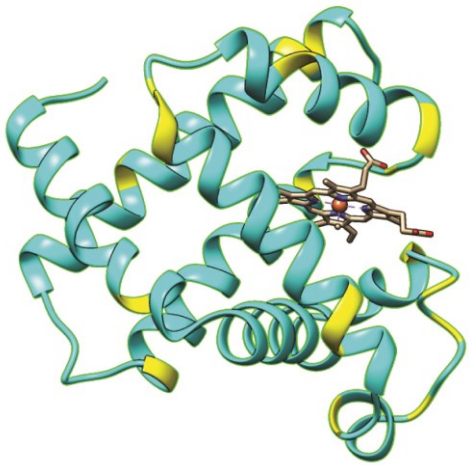


Figure 2: A) Ribbon structure of Mb with coordinated heme group; lysine residues are highlighted in yellow (associated waters and sulfate anion are omitted) (13).

In its typical physiological form the iron cation exits in the iron(II) state. In deoxymyoglobin (deoxy-Mb), the iron(II) cation is coordinated by four pyrrole nitrogens from the heme group and the imidazole nitrogen of His93 in a distorted square pyramidal conformation (5). When oxygen binds to give oxymyoglobin (oxy-Mb), the O_2 molecule serves as the sixth ligand and the Fe^{2+} center shifts into an octahedral conformation (5,11). Iron(II) helps myoglobin fulfill its primary biological function of binding and storing oxygen in the heart and skeletal muscle (11). Myoglobin also coordinates a sulfate anion and a large number of water molecules in a solvent network (12). Although deoxy-Mb is certainly a biologically-relevant species, it proved too difficult to produce and maintain deoxy-Mb under the experimental conditions of this investigation for sufficient time to obtain a well-resolved charge ladder. For this reason, oxy-Mb was the only iron(II) myoglobin species used in this investigation.

The iron center can also be oxidized to its ferric state, yielding metmyoglobin (met-Mb). Iron(II) is rapidly oxidized to iron(III) in free heme molecules or *in vitro* myoglobin (11). Sequestering the heme group within the protein in a physiological context typically maintains the iron(II) state; however, a small degree of met-Mb can occur biologically (5,12). Like ferrous myoglobin, met-Mb coordinates a sulfate anion and a large solvent shell of water molecules (12). Additionally, a water molecule serves as the sixth ligand for the iron(III) center, which prevents oxygen from binding (12). By disrupting its ability to bind oxygen, oxidation to met-Mb inhibits the protein's basic function. Further, the decrease in its net negative charge that occurs (or is assumed to occur) upon oxidation to met-Mb could potentially make Mb more prone to aggregation due to a decrease in the repulsion between like-charged proteins (3,4).

In this study we measured the net charge of oxy-Mb and apo-Mb. In doing so, we quantified the change in charge and the extent of charge regulation that occurs in the transition between these two redox states of myoglobin.

CHAPTER TWO

Materials and Methods

Generating met-Mb Charge Ladders

Although myoglobin in the body typically contains iron(II) (oxy-Mb or deoxy-Mb), lyophilized Mb and Mb in solution have had ample exposure to O_2 in the air occurs in the oxidized form with iron(III), otherwise known as met-Mb. Charge ladders were therefore generated for met-Mb first. Purified met-Mb (Sigma-Aldrich) was dissolved in 100 mM HEPBS buffer, pH 9.0. This myoglobin was divided into two solutions, one of which was reacted with five molar equivalents of acetic anhydride (Sigma-Aldrich) dissolved in dioxane (Sigma-Aldrich). Both solutions were then incubated at room temperature for five minutes of a shaker and then transferred to clean 500 MWCO filter tubes. Buffer exchange was conducted (6000 rpm, 4 °C, 1:5 dilution x 6) to transfer the samples into 10 mM KPO4 buffer at pH 7.40. Samples not used immediately were flash-frozen in liquid N_2 and stored at -80 °C. When used, these samples were defrosted under running water and centrifuged at 33,000 rpm for 30 minutes to remove any aggregated protein.

Analyzing met-Mb Charge Ladders Using CE

Prior to analysis with CE, the concentration of unacetylated and acetylated samples were determined using a UV-VIS spectrophotometer (Beckman; $\varepsilon_{280\ nm} = 13940$

$M^{-1}cm^{-1}$). Samples were diluted to 100 μM and 1 μM dimethylformamide (DMF) was added as a neutral marker. Capillary electrophoresis was then conducted using a Beckman Coulter P/ACE MDQ CE System with a bare fused silica capillary (total length of 60.2 cm, detector length of 50.0 cm). Electrophoresis was conducted at 30 kV and 15 °C, and the absorbance of samples passing the UV-detection window was measured at 214 nm.

After analysis, the migration time of unacetylated met-Mb and each rung in the met-Mb charge ladder was converted to mobility using equation 2,

$$\mu_x = \frac{l_{tot} l_d}{V}\left(\frac{1}{t_x} - \frac{1}{t_{NM}}\right) \qquad (3) \qquad \text{Eq. 2}$$

Where l_{tot} is the total length of the capillary (60.2 cm), l_d is the length from the beginning of the capillary to the detector (50.0 cm), V is the applied voltage (30 kV), t_x is the migration time of species x, and t_{NM} is the migration time of the neutral marker (DMF). The mobility of species x (μ_x), is expressed in units of $cm^2 \cdot kV^{-1} \cdot min^{-1}$. It is important to note that the sign conventions used in this study give myoglobin and its acetylated derivatives, which are all negatively charged and elute after the neutral marker, negative values of μ_x. The mobility of the acetylated Mb derivatives only changes significantly with charge; the greater the number of acetylations, the more negative a protein's charge and the more negative its mobility.

After calculating the mobility of the first several rungs in the met-Mb charge ladder, these values were used to construct a plot of mobility (μ_N) v. acetylation number (N). A linear function was fit to the data for each met-Mb trial using graphing software (with R^2>0.99 for all trials). This relationship can be described by Eq. 3:

$$\mu_N = \frac{e(Z_{Ac(0)} + N\Delta Z_{Ac})}{f_{eff}} \qquad (3) \qquad \text{Eq. 3}$$

$Z_{Ac(0)}$ represents the net charge of unacetylated met-Mb and ΔZ_{Ac} is the change in net charge with each acetylation. For the first several acetylations, ΔZ_{Ac} can be approximated as 0.9. Equation 3 is derived from equation1 by replacing Z with $Z_{Ac(0)} + NZ_{Ac}$: that is, the net charge of each acetylated derivative is the sum of the net charge of unmodified met-Mb and the change in net charge per acetylation multiplied by the number of acetylations. By rearranging and simplifying equation 3, the x-intercept of this plot can be expressed as:

$$N_{int} = -\frac{Z_{Ac(0)}}{\Delta Z_{Ac}} \qquad \text{Eq. 4}$$

The x-intercept, N_{int}, is equal to the theoretical number number of acetylations that would be necessary for the mobility, and thus the net charge, of met-Mb to be zero (since the charge ladder eliminates hydrodynamic drag as a factor). Rearranging equation 4 yields:

$$Z_{Ac(0)} = -\Delta Z_{Ac} N_{int} = -0.9 N_{int} \qquad \text{Eq. 5}$$

The x-intercept from each trial could be determined from the parameters of the calculated linear fit; this value was used to calculate $Z_{Ac(0)}$ for each trial; these values were averaged to determine the net charge of unmodified met-Mb.

Generation and Analysis of oxy-Mb Charge Ladders

Charge ladders of oxy-Mb were generated by reducing the met-Mb sample. A 1.0 M solution of sodium dithionite, $Na_2S_2O_4$, was prepared in ultrapure water. Immediately prior to each run, 100 molar equivalents of sodium dithionate were added to the met-Mb solution to reduce it to oxy-Mb. The oxidation state of the Mb sample was verified using UV-VIS spectroscopy, as each Mb species has a distinct λ_{max} (Fig. 3). A UV-VIS spectrum was taken of the Mb sample immediately prior to and immediately following

each run to ensure that the electropherogram taken actually represented oxy-Mb. The same CE conditions used for met-Mb were used when analyzing oxy-Mb. The same process was used to calculate the mobility from migration time for unacetylated oxy-Mb and each rung in the oxy-Mb charge ladder.

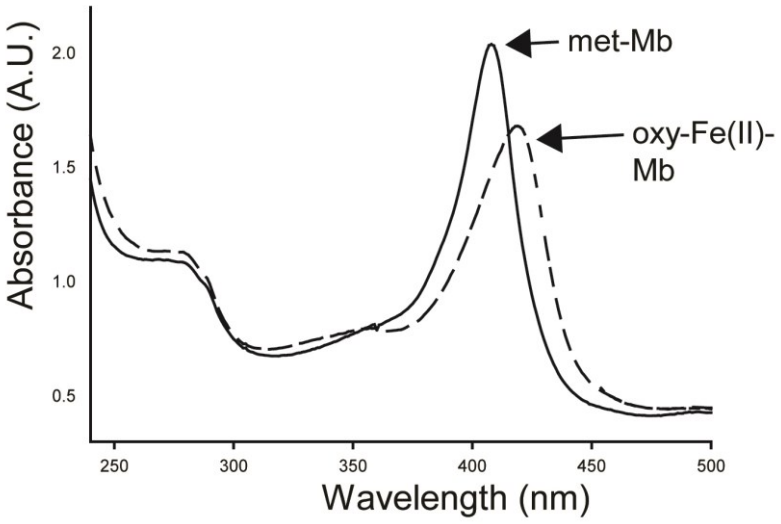


Figure 3: UV-VIS spectra of oxy-Mb and met-Mb

Theoretical Net Charge Calculations

The theoretical net charge of oxy-Mb and met-Mb was determined using the general strategy outlined in the previous chapter. Myoglobin's peptide sequence was obtained from reference 14 and analyzed with an online Protein Calculator (15). The Protein Calculator used the known pK_a values of each individual amino acid to calculate the extent of protonation (and thus the charge) of each ionizable residue a pH 7.40. It then summed these values to estimate the net charge of the 154 residue polypeptide. The charges of the sulfate anion (-1) and the iron cation (+2 or +3) that myoglobin

coordinates were then added to the charge of the polypeptide to give the predicted net charge values for oxy-Mb and met-Mb.

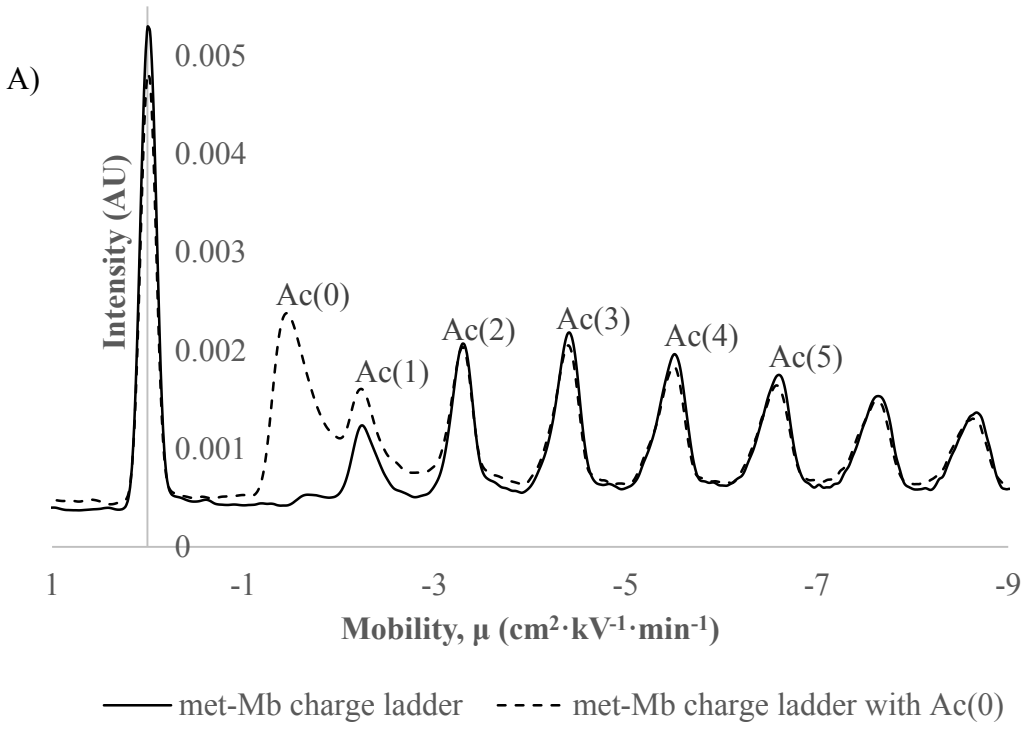
CHAPTER THREE

Results and Discussion

Net Charge of met-Mb

Protein charge ladders were generated for met-Mb and analyzed using CE. Several of these charge ladders were spiked with unacetylated met-Mb in order to establish a reference point. Two typical met-Mb charge ladders, one spiked with Ac(0) and one without, are shown in Fig. 4A. (For reference, overlaid electropherograms of all of the met-Mb charge ladders are given in Figure S2.) The peak corresponding to unacetylated met-Mb peak is labeled Ac(0) in Fig. 4A; the rest of the peaks in the met-Mb charge ladder are labeled accordingly as Ac(N), where N represents the number of acetylations. For each charge ladder, the mobility (μ) of each acetylated species was plotted against the number of acetylations. The μ v. Ac(N) plot corresponding to the met-Mb charge ladder from Fig. 4A is shown in Fig. 4B. A linear equation was fit to the data, the x-intercept was extrapolated, and the net charge of unmodified met-Mb was calculated as described in the previous chapter; this procedure was followed for each of 12 trials. These values were averaged and the net charge of met-Mb was determined to be -1.02 ± 0.06 (Table 1).

The calculated net charge for myoglobin at pH 7.40 differs from the theoretical estimate at this pH, +3.0, by 4.02 ± 0.06 units. This reinforces the limitations to the traditional assumptions underlying net charge calculations and reinforces the need for experimental quantification.



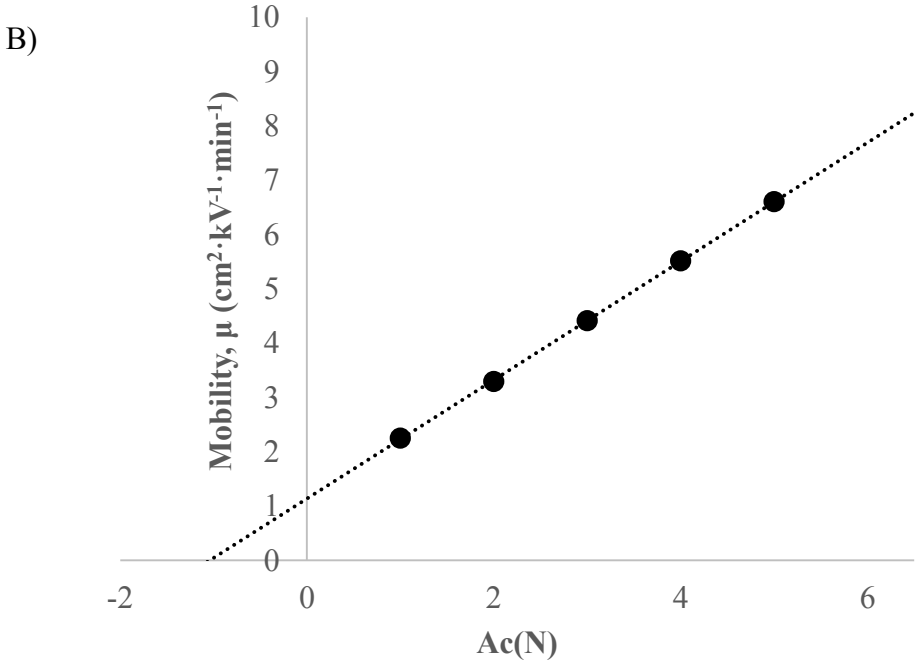


Figure 4: A) Electropherograms of met-Mb, and B) Mobility v. Ac(N) plot for met-Mb.

Net Charge of oxy-Mb

The met-Mb charge ladder was reduced to oxy-Mb using sodium dithionate. A typical charge ladder is shown in Figure 5A (all overlaid trials are given for reference in Figure S3). UV-VIS spectra were taken before and after each reduced trial to verify the oxidation state of the myoglobin. Sample spectra are given in Figure 3 for reference. As with the met-Mb charge ladder, each peak in the oxy-Mb charge ladder has been labeled with its acetylation number using Ac(0) as a reference. The mobility of each rung of the oxy-Mb charge ladder has a higher mobility than the corresponding rung in the met-Mb ladder, indicating that oxy-Mb is more negative than met-Mb. Following conventional net charge calculations, the oxy-Mb is expected to be a single unit more negative than met-Mb, as the heme iron is reduced from iron(III) in met-Mb to iron(II) in oxy-Mb.

For each oxy-Mb trial, a plot of mobility v. Ac(N) was constructed and the net charge of the unmodified protein was extrapolated as for met-Mb (Fig. 5B). The 3 trials of oxy-Mb were averaged to yield a net charge of -1.67 ± 0.09 units (Table 1).

	Theoretical [a]	**Experimental**
met-Mb	+3.0	-1.02 ± 0.06
oxy-Mb	+2.0	-1.67 ± 0.09

Table 1: Net charge of myoglobin in various redox states.

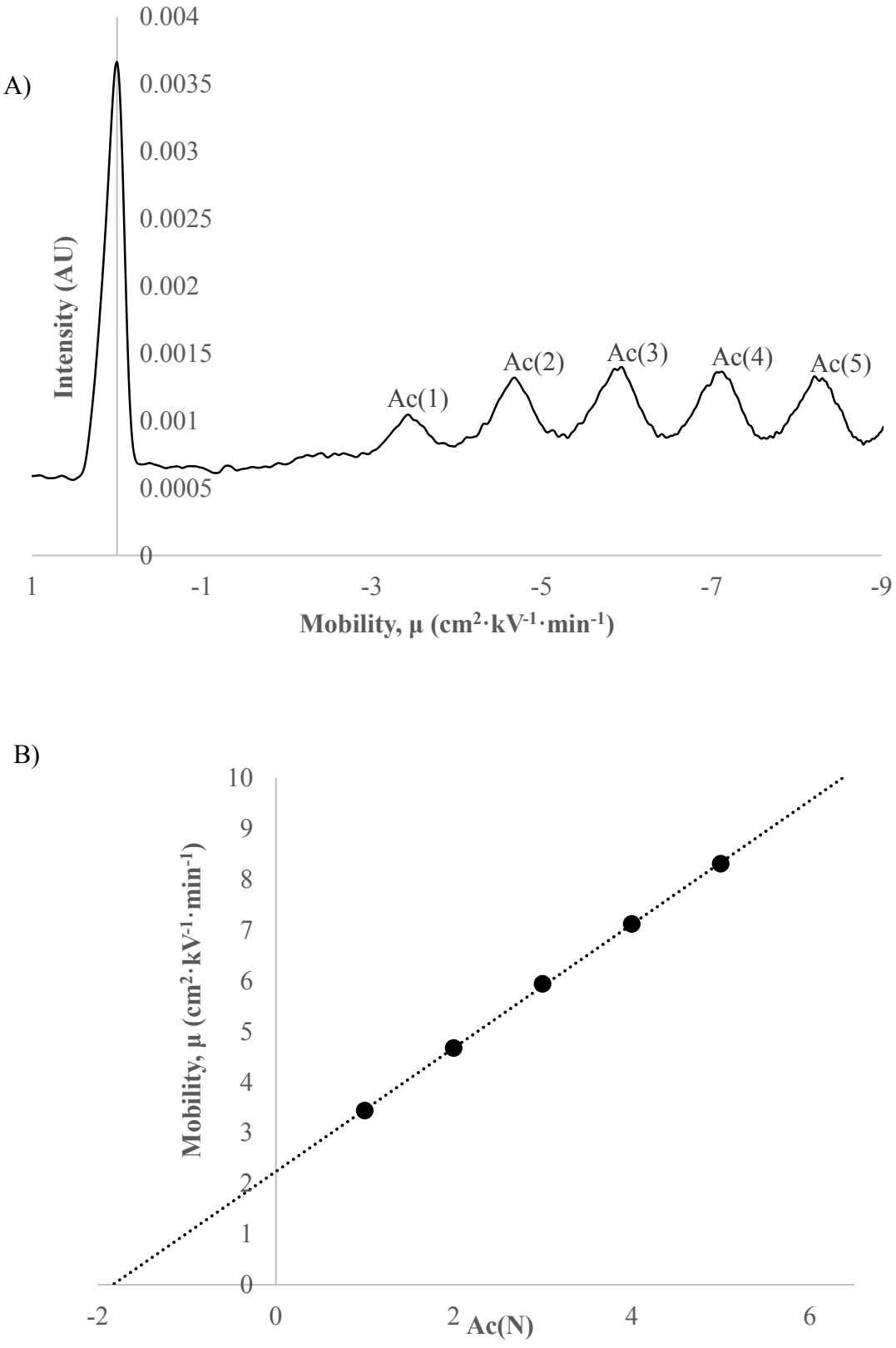


Figure 5: A) Electropherogram for oxy-Mb, and B) Mobility v. Ac(N) plot for oxy-Mb.

Comparison	Value
Theoretical difference	1.0
Experimental difference	0.6 ± 0.1
Charge Regulation	0.4 ± 0.1

Table 2. Comparison of the net charge of oxy-Mb and met-Mb.

Charge Regulation in Myoglobin

The experimentally-determined net charge of met-Mb and oxy-Mb (Table 1) indicate that charge regulation occurs in myoglobin. A comparison of the net charges of oxy-Mb and met-Mb is given in Table 2. Charge regulation is quantified as the difference between the theoretical and the experimental net charge; between oxy-Mb and met-Mb, the net charge changed by 0.4 ± 0.1 units less than expected between oxy-Mb and met-Mb. The error in this measurement is worth noting (~30%), and is likely due to the difficulty in obtaining a high-quality charge ladder of oxy-Mb. The reason for this difficulty is not certain; it is possible that the high concentration of dithionite interfered with the UV detection, or that the conversion from met-Mb to oxy-Mb was incomplete resulting in much lower, broader peaks than those seen in the met-Mb charge ladder. Nonetheless, a conclusive discrepancy was observed between the expected change in net charge and the actual change that occur.

CHAPTER FOUR

Conclusion

Myoglobin and Net Charge

The net charge of oxy-Mb and met-Mb were measured experimentally using protein charge ladders and capillary electrophoresis. Not only did the experimental values differ by ~4 units from the predicted values, but the sign of the net charge was also different. Whereas the calculations based on textbook pK_a values for each residue and the cofactors' formal charges yielded small positive values, the measured net charge of each Mb species were small and negative. Although these predicted values are generally understood to be estimates, this investigation demonstrated that, in the case of myoglobin, these "estimates" were substantially different from the actual net charge. In the case of met-Mb, the prediction differed from the actual net charge by 4.02 ± 0.1 units, or ~400%. This reinforces the importance of experimentally calculating protein net charge rather than relying on predictions.

Charge Regulation in Mb Redox States

This investigation verified the existence and quantified the extent of charge regulation in the transition between oxy-Mb and met-Mb. When the heme iron is oxidized from the biologically-prevelant iron(II) to iron(III), the net charge changes by 0.4 ±0.1 units less than expected. Myoglobin was observed to regulate its net charge upon oxidation in a way that decreased the overall change.

The mechanism whereby this charge regulation occurs is not currently known. However, one possibility involves the water molecule that serves as the sixth ligand for iron(III) in met-Mb (replacing the O_2 ligand in oxy-Mb). It is generally difficult to resolve protons in X-ray crystallography, so it is possible that the observed H_2O is actually OH^- or H_3O^+. Coordinating a hydroxide anion is one possible mechanism by which myoglobin might regulate its net charge upon oxidation to met-Mb. Myoglobin's ionizable residues exist in a co-dependent, nonlinear network in which the pK_a (and thus the extent of ionization) of each residue is affected by the charged moietie s around it, including other charged residues as well as tightly-bound cofactors. Both the increase in the charge of the iron and the potential coordination of a hydroxide group would affect this network in ways that are still difficult to predict. Experimentally measuring the net charge of myoglobin in different redox states is a start in understanding the process of charge regulation.

The biological rationale behind charge regulation is still being elucidated, but several possibilities exist. Charge regulation could be a strategy to minimize adverse effects of significant changes to net charge, including the loss of functions that rely to some extent on charge (e.g. interaction with other charged cofactors, ligands, or other charged biomolecules) and/or the gain of adverse function (e.g. aggregation). It is difficult to hypothesize the precise function of charge regulation when the role of net charge is still incompletely understood. However, a better understanding of the range and extent of charge regulation will likely provide insight into the overall role that net charge plays in proteins.

This investigation demonstrated the existence of charge regulation between oxy-Mb and met-Mb and serves as a starting point for studying the extent, mechanism, and role of charge regulation in myoglobin. The extent of charge regulation in metalloproteins is not known, and the phenomenon itself is still poorly understood. By demonstrating the existence of charge regulation in myoglobin, this investigation provides a small piece of this complex but emerging puzzle.

APPENDIX

APPENDIX

Supplemental Information

MS of Unacetylated Met-Mb and Met-Mb Charge Ladder

The mass spectra seen in Fig. 6 give the mass of unmodified met-Mb and the mass of each acetylated derivative. The mass of unacetylated Mb is 16951 Da and each peak in the mass spectrum of the met-Mb charge ladder differs by the ~42 Da expected by the addition of an acetyl group (1). Although this spectrum does not provide any information about net charge, it does provide information as to the identity of each species in the charge ladder and verifies the assigned acetylations. Note that while the MS data shows a total of seventeen acetylations, only five were used in the net charge calculations because the linear relationship between Ac(N) and mobility breaks down after the first several rungs.

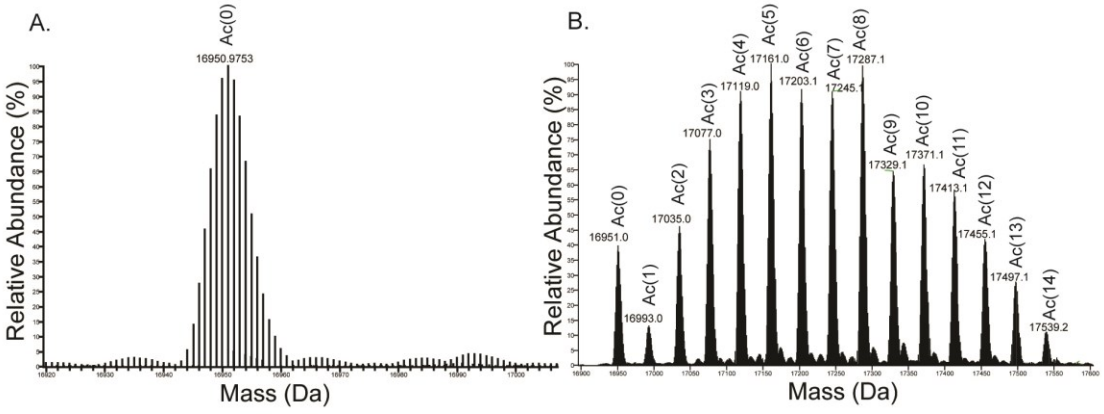


Figure S1: Mass spectra of A) Ac(0) met-Mb, and B) the met-Mb charge ladder.

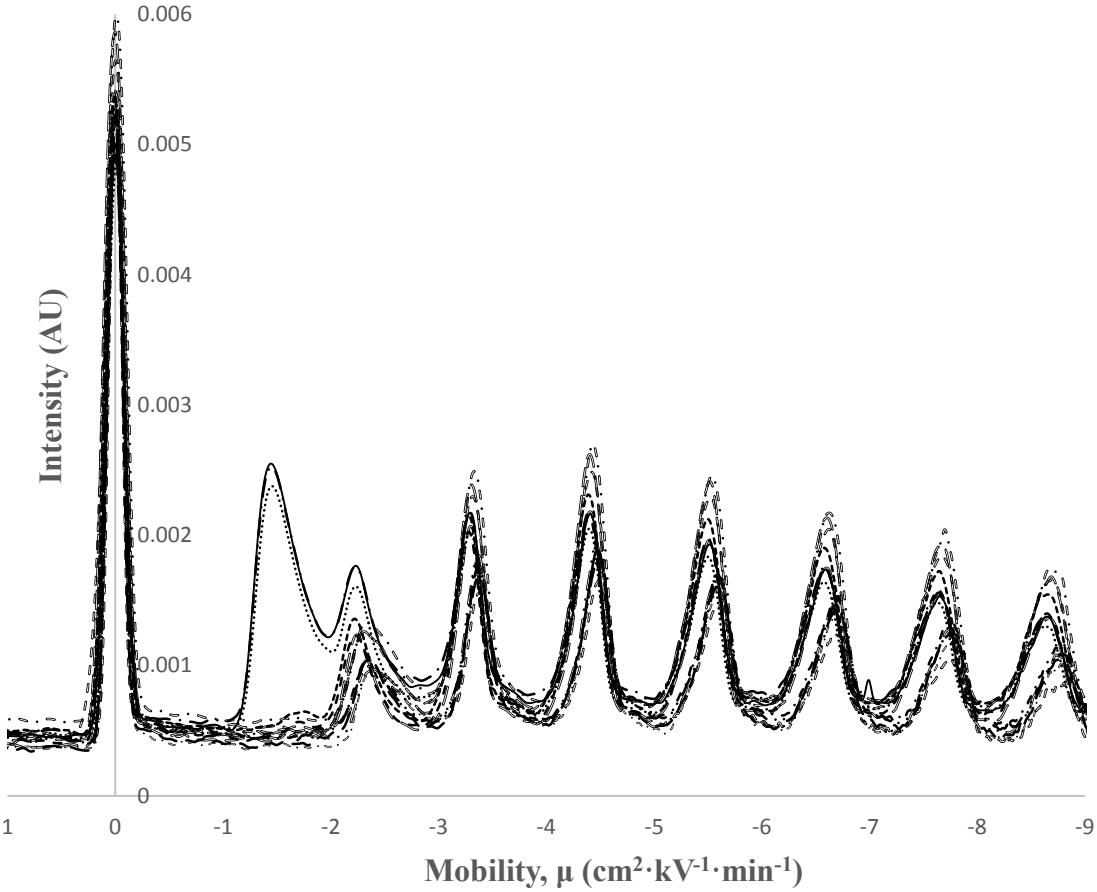


Figure S2: Overlaid electropherograms of all met-Mb trials. All trials without extra Ac(0) added (12 trials) were used to calculate the net charge value given in Table 1.

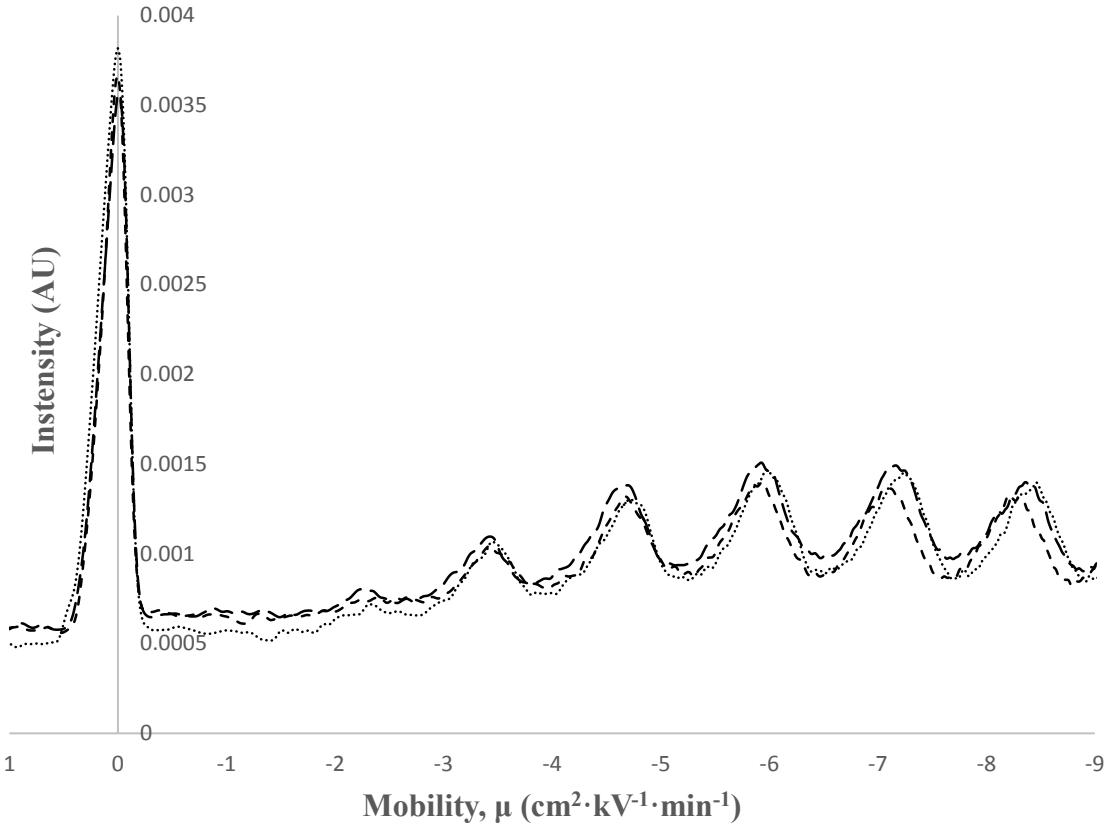


Figure 8: Overlaid traces of all oxy-Mb trials. All three trials were used to calculate the net charge value given in Table 1.

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