pH TITRATIONS OF MOLLUSCAN PARAMYOSIN AT TWO DIFFERENT IONIC STRENGTHS

L. B. COOLEY AND S. KRAUSE, *Department of Chemistry, Rensselaer Polytechnic Institute, Troy, New York 12181 U.S.A.*

ABSTRACT Paramyosin extracted from the adductor muscle of *Mercenaria mercenaria*, the chowder clam, was titrated both in 0.3 M KCl and in 1 mM KCl. Both the presumed native form of the molecule, acid-*R*-paramyosin, and a slightly degraded form, *β*-paramyosin, were studied. Titrations of both types of paramyosin were similar in 1 mM K^+, except that the native paramyosin is more highly charged at pH 3.2 than *β*-paramyosin, as postulated previously (DeLaney and Krause, 1976, *Macromolecules*, **9**:455), and that more groups titrate on the native molecule than on *β*-paramyosin, both between pH 3.2 and 3.3 and between pH 3.2 and 10. Titrations in 0.30 M KCl, unlike those in 1 mM K, depended on starting pH; long term exposure to alkali solutions during dialysis, previously shown to cause partial dephosphorylation of paramyosin (Cooley et al., 1979, *J. Biol. Chem.*, **254**:2195), apparently also leads to a change in intermolecular interactions sufficient to cause changes in the titration curves in 0.30 M KCl but not in 1 mM K^+.

INTRODUCTION

Paramyosin is a large, rod-like protein which has been isolated from many invertebrate muscles. This protein is particularly abundant in those molluscan muscles capable of "catch," which is the ability of these muscles to remain in the contracted state for long periods of time with little or no expenditure of energy.

It has been suggested that the paramyosin molecule may play a unique role in the catch mechanism (Johnson et al., 1959; Ruegg, 1961 and 1971). As early as 1959, Johnson et al. (1959) noted that small changes in pH and ionic strength had a significant effect on isotonic shortening of glycerinated catch muscles. They also noted that paramyosin in vitro underwent parallel changes in solubility as the pH and ionic strength were changed. This led them to suggest that small changes in pH and ionic strength in vivo may result in changes in the aggregation of paramyosin molecules in the thick filaments of the catch muscle. An increase in aggregation might then be responsible for locking the catch muscle in a contracted state and maintaining tension, without additional energy expenditure. A decrease in intermolecular interactions after another change in pH and ionic strength would then result in a release of muscle tension.

The paramyosin most commonly studied in vitro is from the adductor muscle of *Mercenaria mercenaria*. Unfortunately, differences in sample preparation and handling of paramyosin from this source often result in partial degradation of the molecule (Stafford and Yphantis,

Dr. Cooley is recipient of a fellowship from Corning Glass Works.

Dr. Krause is recipient of a Research Career Award from National Institutes of Health.

1972), which complicates the interpretation of data collected before 1972. It is now known (Stafford and Yphantis, 1972) that native or undegraded paramyosin from *M. mercenaria* adductor muscles has a molecular weight of 2.10×10^5; this molecule will be designated α-*R*-paramyosin (Stafford, 1973) or acid-*R*-paramyosin (Cooley, 1978), depending on details of the extraction procedure. The first degraded type of paramyosin, 2.00×10^5 mol wt, will be designated β-paramyosin (Stafford, 1973), and the second degraded type of paramyosin, 1.87×10^5 mol wt, will be designated γ-paramyosin.

In general, β-paramyosin, which is missing a 10^4 mol wt segment from the C-terminal end of native paramyosin (Stafford and Yphantis, 1972), is significantly more soluble in water than α-*R*-paramyosin in the ionic strength range from 0.1 to 0.4 M and in the pH range from 6.0 to 8.0, in which most investigations have been performed (Merrick, 1975; Merrick and Johnson, 1977; Melson and Cowgill, 1976; Cooley et al., 1979). In addition, differences between the aggregation behavior of α-*R*-paramyosin and of β-paramyosin in the pH range of 3 to 10 in 1 mM buffer were observed by Krause and DeLaney (1977), using transient electric birefringence techniques. Both molecules appeared to aggregate as staggered dimers at a pH above 7; at higher pH values, side-to-side aggregation of other molecules onto these staggered dimers was postulated, until, at pH 10, only monomers were observed. The largest aggregates, containing about seven molecules, were observed at pH 8.8–9.0 in α-*R*-paramyosin solutions; the same types of aggregates were observed at pH 7.8 in β-paramyosin solutions. These differences in aggregation behavior were attributed to differences in charge and charge distribution between α-*R*-paramyosin and β-paramyosin in the appropriate pH ranges. Such charge differences were expected to have an effect on the titration behavior of the molecules at 1 mM ionic strength.

DeLaney and Krause (1976) also observed differences between monomeric α-*R*-paramyosin and monomeric β-paramyosin in 1 mM buffers at low pH. At pH 3.2, α-*R*-paramyosin was observed to have a higher specific Kerr constant (K_{sp}) than β-paramyosin, as measured by transient electric birefringence. This indicated that α-*R*-paramyosin either has a greater dipole moment or a greater charge anistropy than β-paramyosin at this pH. At the same pH, the length of the α-*R*-paramyosin molecule was calculated to be ~6% greater than that of β-paramyosin using the observed relaxation time of the birefringence. However, as the pH increased from pH 3.2 to 3.3, both the K_{sp} and the relaxation time of α-*R*-paramyosin decreased to the values observed for β-paramyosin. DeLaney and Krause explained these results by postulating the presence of extra (positively) charged groups on the extra piece present in α-*R*-paramyosin. As the pH increases, uncharged carboxylic acid groups nearby titrate so that their negative charges can neutralize the positive charges on the extra groups. Thus, both the K_{sp} and the dipole moment of α-*R*-paramyosin would be expected to decrease. Furthermore, it was postulated that the extra piece becomes more flexible as the net charge on it decreases, resulting in a decrease in the length of the rigid part of the molecule; this decrease in length would explain the decrease in the observed birefringence relaxation time.

This present study was an attempt to test the hypotheses of Krause and DeLaney (1977) and DeLaney and Krause (1976) by titrating the native molecule, acid-*R*-paramyosin, and β-paramyosin in 1 mM KCl between pH 3 and pH 10. In addition, both the native molecule and β-paramyosin were titrated in 0.3 M KCl for comparison with the very contradictory titrations reported previously in the literature (Johnson and Kahn, 1959; Riddiford and

Scheraga, 1962). Although these earlier titrations were almost certainly both performed on slightly degraded molecules, they were very different from each other, and it was interesting to determine why.

METHODS AND MATERIALS

Protein Preparation

Because acid-*R*-paramyosin and α-*R*-paramyosin have the same molecular weight and similar, if not identical, physical properties (Merrick, 1975; Cooley, 1978), and because the yield of acid-*R*-paramyosin per gram of adductor muscle is 3 to 5 times greater than that of α-*R*-paramyosin, acid-*R*-paramyosin was usually used as the native molecule in this study.

Acid-*R*-paramyosin was prepared according to the acid extraction method of Hodge (1952) as modified by Edwards et al. (1977), but with 0.5 mM dithiothreitol (DTT) included in all solutions to insure that the extracted protein was in the reduced state (Stafford, 1973). β-Paramyosin was prepared using the ethanol extraction of Johnson et al. (1959) with a short-term 1-h extraction, as suggested by Stafford and Yphantis (1972). α-*R*-Paramyosin was prepared in the same way as β-paramyosin, except that 0.01 M EDTA and 0.5 mM DTT were included in all solutions, also as suggested by Stafford and Yphantis (1972).

All samples were found to be monodisperse by using a long-term sodium dodecyl sulfate gel electrophoresis similar to that described by Yeung and Cowgill (1976), which uses a recirculating gel buffer. Phosphorylase *a* was used as a standard; acid-*R*-paramyosin and α-*R*-paramyosin migrated more slowly than phosphorylase *a*, and β-paramyosin migrated at the same rate as phosphorylase *a*, as previously reported by Merrick (1975). The OD_{280}/OD_{260} ratios of the final samples were found to be >2.0 (usually 2.3–2.4), indicating a low nucleotide contamination. Sample concentration was determined spectrophotometrically using a value of $E^{1\%}_{1cm} = 3.125$ at 277 nm for all samples (Gaffin, 1967).

Titration Procedure

Standard solutions of HCl and KOH were prepared approximately 1.0 N using reagent grade HCl or KOH and freshly boiled, deionized water. The HCl solution was standardized against anhydrous Na_2CO_3 using the mixed indicator bromocresol green and methyl orange. The KOH solution was standardized against potassium acid phthalate with phenolpthalein as an indicator. Both solutions were stored under nitrogen in Nalgene bottles and diluted volumetrically with freshly boiled dionized and distilled water to the necessary concentration just before use.

Samples of paramyosin to be titrated at low ionic strength were diluted to 2–3 mg/ml and then dialyzed against either 0.89 mM KCl + 1 mM HCl, giving a pH of ~3, or 0.89 mM KOH, giving a pH of ~10, for 24 h at 4°C. A high speed centrifugation was used to finally clarify the solution. The low pH protein solutions were titrated with 0.000886 M KOH while the high pH protein solutions were titrated with 0.00089 M KCl + 0.00102 M HCl. Whenever an acid-*R*-paramyosin sample was titrated, all solutions contained 0.5 mM DTT. Paramyosin samples to be titrated at high ionic strength were treated similarly except that they were dialyzed for 24 h at 4°C against either 0.39 M KCl + 0.0102 M HCl or against 0.29 M KCl + 0.01 M KOH. The low pH protein solutions were titrated with 0.29 M KCl + 0.0106 M KOH while the high pH solutions were titrated with 0.30 M KCl + 0.0102 M HCL.

The 5-ml samples were titrated under flowing nitrogen in a water-jacketed cell maintained at 20 ± 1°C using a temperature controlled circulator. Solutions were stirred constantly using a magnetic stirrer. A calibrated microprobe combination electrode (Fisher Scientific Co., Pittsburgh, Pa.) suitable for use in the pH range 0–14 was used for the high ionic strength titrations, and a probe combination pH electrode (Beckman Instruments, Inc., Fullerton, Calif.) made of general purpose glass was used for the low ionic strength titrations. A Fisher Scientific Accumet (model 520) digital pH meter capable of monitoring pH values to ± 0.001 pH units was used to measure the pH of the protein solutions. The pH meter was standardized at the beginning of each day and rechecked at the end using pH Hydrion

standard buffers available from Fisher Scientific. Small additions of titrant were added slowly using a calibrated micrometric syringe buret to minimize exposing the protein to large amounts of strong acid or base. The amount of titrant varied from 0.05 to 0.50 ml, depending on the pH region, so that the change in pH was always <0.1 pH unit. The pH was judged stable when it no longer varied noticeably in the 0.001 pH unit; this took from <1 min to several hours, depending on the pH region. As might be expected, stable pH took longer to attain in those pH regions in which the molecules aggregated or precipitated. Complete titrations lasted up to 3 d. When necessary, solutions were stored overnight in a refrigerator under nitrogen and under a microabsorption tube containing the carbon dioxide absorbent, Ascarite; SDS gel electrophoresis showed no degration of a sample during a 3-d titration at 20°C. After the sample titration was complete, the same procedure was used for a blank titration. The blank consisted of 5 ml of the last dialysis solution.

This procedure is somewhat unusual for four reasons. First, one normally determines the isoionic point of a protein by passing a solution containing the protein through a mixed resin column to deionize the solution. Then a specific amount of salt is added to this solution to give the desired ionic strength. This procedure is not possible with paramyosin because it is insoluble in the region of the predicted isoionic point, pH 5–6 (Szent-Györgyi, 1960). Secondly, the protein concentration should be 1% or more; we used solutions containing 0.2–0.3% protein, because the protein solution becomes extremely viscous and difficult to handle at higher concentrations. Even gentle stirring of concentrated protein solutions results in foaming or small fiber formation. However, since paramyosin contains many titratable groups it is relatively easy to measure the number of groups titrating, even at a low protein concentration. Thirdly, although one should keep the ionic strength constant during a titration, we kept the K^+ concentration constant. At the high ionic strength, with no buffers, and no small ions with a charge greater than ± 1 present, the ionic strength is approximately the same as the K^+ concentration. For consistency, the same method was used at low ionic strength. Finally, it is normal to titrate from an intermediate pH to an acidic or basic endpoint and then to reverse the direction of titration and to titrate back to the starting pH. This generally ensures that the titration is reversible and in equilibrium at all points. However, when

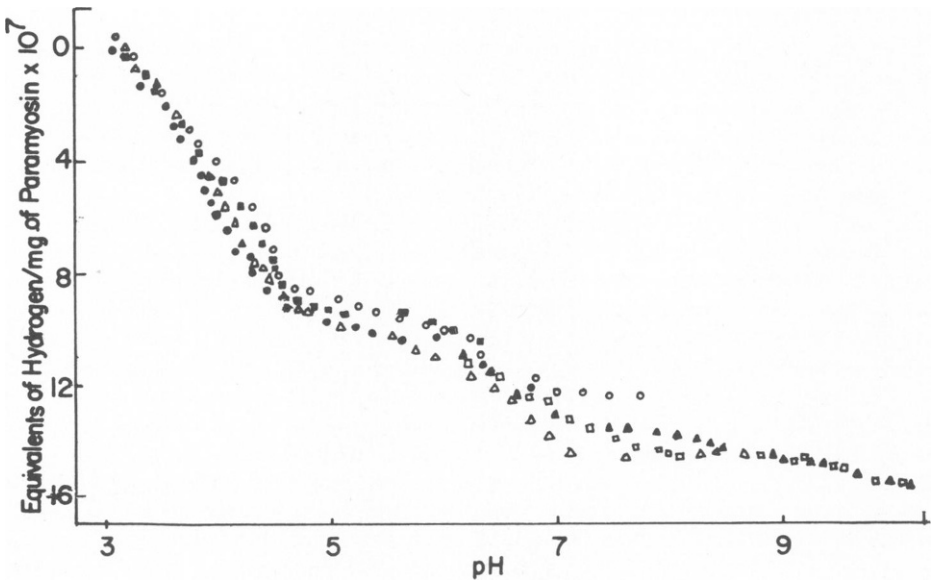

FIGURE 1 Titration of acid-*R*-paramyosin in 0.89 mM K^+. Different symbols indicate representative points from different titrations. Titrations started in base (▲, □) are superimposed on those titrations starting in acid, (△, ○, ●, ■), "Equivalents of hydrogen" refer to numbers of protons titrated from an arbitrary starting point near pH 3.

this was attempted with paramyosin in 0.3 M K^+, nonreproducible portions of the titration curve were discovered. Therefore, separate titrations were performed, one starting at low pH and titrating to a basic endpoint and one starting at high pH titrating to an acid endpoint. It was found that in 1 mM K^+, the two titrations were superposable, whereas at 0.3 M K^+ the two titrations were not superposable. The implications of this irreversibility at high ionic strength and the difference between the results at the two ionic strengths will be considered below.

Titration Calculations

The number of equivalents of H^+ bound to the protein was calculated as described by Tanford (1962), using the blank titration and assuming additivity of the volumes of titrant with protein solution. For the titrations starting a low pH, the number of equivalents of H^+ bound was divided by the number of milligrams of protein present and then plotted as a function of pH, assuming that no H^+ was bound at the starting pH. The equivalents of H^+ bound per milligram of protein starting at high pH were also plotted as a function of pH and superimposed on the titrations starting in acid.

RESULTS

1 mM KCl

The titrations of both acid-*R*-paramyosin and of β-paramyosin started at high pH were superposable on those starting at low pH, within experimental error, as shown in Fig. 1. For comparison, the titration curves of acid-*R*-paramyosin and of β-paramyosin are superposed in Fig. 2 at the calculated isoelectric point, pH 5.8 (Riddiford and Scheraga, 1962). In general, the titration of acid-*R*-paramyosin was less reproducible than that of β-paramyosin, as indicated by the length of the slashes in Fig. 2, This is not unexpected, since the native protein is more viscous, harder to handle, and more aggregated than β-paramyosin.

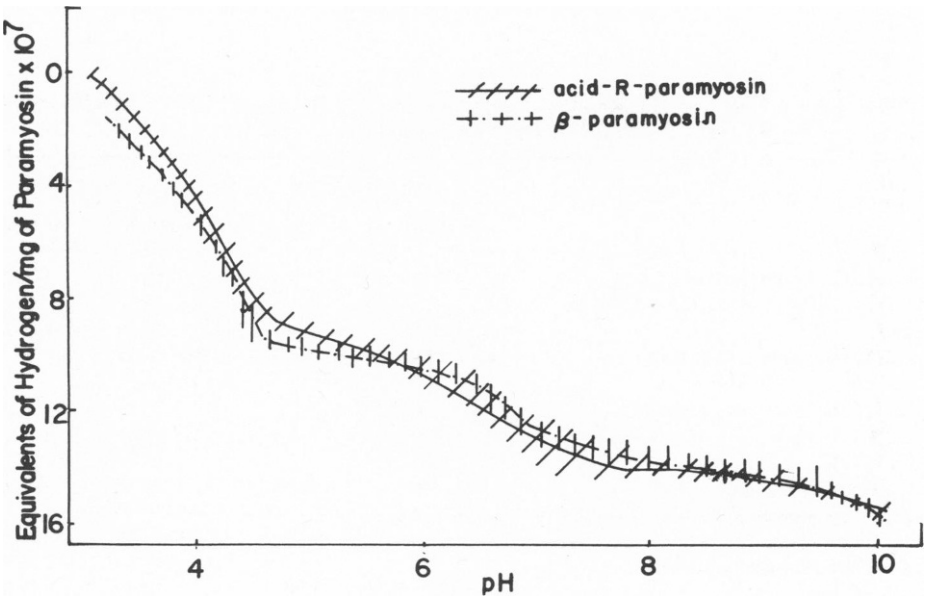

FIGURE 2 Titrations of acid-*R*-paramyosin and β-paramyosin in 0.89 mM K^+ superimposed at pH 5.8. Slashes indicate scatter of points for each titration. "Equivalents of hydrogen dissociated" refer to numbers of protons titrated from an arbitrary starting point near pH 3.

Acid-*R*-paramyosin was insoluble between pH 4.3 and ~6.9; *β*-paramyosin was insoluble over a somewhat smaller pH range, pH 4.3–6.6. According to Steinhardt and Harris (1940), insoluble proteins usually bind very few H^+ ions in the neutral pH region. This generalization seems to be true for both acid-*R*-paramyosin and for *β*-paramyosin between pH 4.5 or 4.6 and pH 6.0. But above pH 6.0 (to ~pH 7.0) the number of groups titrating increased significantly, even though the protein was still insoluble.

The usual quantitative analyses of titrations are difficult to perform at this low ionic strength due to an increase in electrostatic interactions (Tanford, 1962). It should, however, be possible to relate differences in the observed behavior of native paramyosin and *β*-paramyosin to differences in the calculated charges on the molecules as obtained from the average titration curves shown in Fig. 2 with an assumed isoelectric point of pH 5.8 (Riddiford and Scheraga, 1962). As shown in Table I, the calculated charge on acid-*R*-paramyosin at the precipitation point, pH 4.3, was +87; the charge on *β*-paramyosin at its precipitation point was +52. Similarly, the charge on acid-*R*-paramyosin at pH 6.9, where it resolubilized, was higher than on *β*-paramyosin at pH 6.6, where it resolubilized: −49 vs. −13, respectively. In fact, the total number of groups titrating on acid-*R*-paramyosin while it was insoluble was about double the number that titrated on *β*-paramyosin: 134 vs. 92. This is unexpected, because acid-*R*-paramyosin is only ~5% larger than *β*-paramyosin. Furthermore, the total number of groups titrating on acid-*R*-paramyosin between pH 3 and pH 10 was significantly larger than on *β*-paramyosin; 319 groups titrated on acid-*R*-paramyosin, whereas only 280 titrated on *β*-paramyosin. Nevertheless, the charge on the molecules appeared to be the same at pH 10 (Fig. 2). The greatest difference in titration behavior as measured by the calculated charge per molecule seemed to occur below the isoelectric point of pH 5.8. Furthermore, more groups appeared to be titrating on the native molecule than on

TABLE I
NUMBER OF GROUPS TITRATING AND CALCULATED CHARGE OF PARAMYOSIN AT SELECTED pH VALUES IN 0.89 mM K^+

	Acid-*R*-paramyosin		*β*-Paramyosin	
	Number	pH range	Number	pH range
Total number of groups titrating	319	3.2–10.0	280	3.2–10.0
Number of groups titrating in precipitation region	134	4.3–6.9	92	4.3–6.6
Number titrating pH 3.20–3.30	5.2* ± 1.4‡	3.20–3.30	3.2 ± 1.2‡	3.20–3.30
Calculated charge on the molecule§				
at pH 3.2	+209		+175	
at pH 10.0	−108		−107	
at precipitation point	+87; −49	4.3; 6.9	+70; −13	4.3; 6.6
when very large aggregates of 100 monomers exist	−8 to −63	6.0–7.3	−3 to −14	6.0–6.5
at pH of maximum aggregation in alkaline solution	−88	9.0	−66	7.8

*Number titrating on *α*-*R*-paramyosin is 8.4 ± 2.0.
‡Standard deviation.
§Using the average titration curves in Fig. 2 and assuming the isolectric point is pH 5.8.

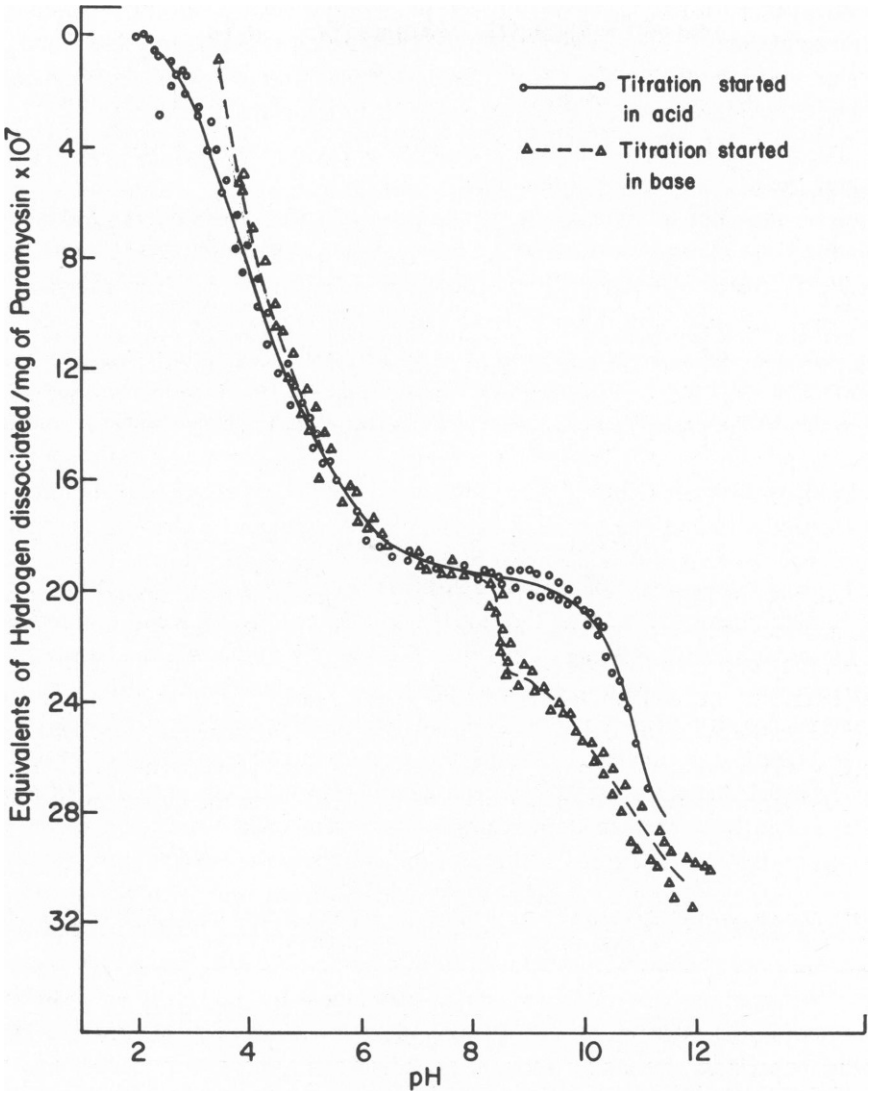

FIGURE 3 Titration curves of acid-*R*-paramyosin in 0.30 M KCl. Points shown on the curve are representative of many points taken on at least two trials for each starting pH.

β-paramyosin between pH 3.2 and pH 3.3 (Table I); 5.2 ± 1.4 groups on acid-*R*-paramyosin, 8.4 ± 2.0 groups on α-*R*-paramyosin, but only 3.2 ± 1.4 on β-paramyosin.

0.30 M KCl

The titration curves for native paramyosin in 0.30 M KCl starting in acid and starting in base are shown in Fig. 3. Unlike the titrations in 1 mM K^+, the titrations in 0.30 M KCl are not superposable. The curve starting in acid has the shape expected for a protein with an amino acid content similar to paramyosin and with no unusual changes in conformation or aggregation during the titration. On the other hand, the curve starting in base showed many groups titrating between pH 9 and 8. In this pH region, the rate of titration was extremely slow; sometimes an hour or more was required to obtain a stable pH reading.

Such a slow titration involving many titrating groups usually indicates either a change in conformation or aggregation (Tanford, 1962).

When the titrations were performed with β-paramyosin, an almost identical set of curves was obtained, showing that the irreversibility was a function of starting pH and not a pecularity of the native molecule. As a matter of fact, the titration curves of both acid-*R*-paramyosin and β-paramyosin starting in acid are very similar to the titration curve, probably of β-paramyosin, starting at pH 7, reported by Riddiford and Scheraga (1962). A semiempirical thermodynamic analysis in the manner of Linderstrøm-Lang as described by Tanford (1962) confirmed that acid-*R*-paramyosin and β-paramyosin titrate similarly when the titrations are started in acid, and also that both types of paramyosin titrate similarly to β-paramyosin starting at pH 7, as reported by Riddiford and Scheraga (1962).

Regardless of the direction of the titration, the titrations of acid-*R*-paramyosin and β-paramyosin showed that very few groups seem to be titrating in the pH region where paramyosin changes from being completely insoluble to completely soluble. More specifically, acid-*R*-paramyosin undergoes this change between pH 7.2 and 6.2; in this region 29 groups were titrating. On the other hand, β-paramyosin had 26 groups titrating between pH 6.7 and 6.2, where it underwent the change from soluble to insoluble.

DISCUSSION

Comparison of High Ionic Strength Titrations with Literature Titrations

Although the titration curves of both acid-*R*-paramyosin and β-paramyosin in 0.30 M KCl starting in acid are very similar to the titration curve reported by Riddiford and Scheraga (1962) for β-paramyosin starting at pH 7 in 0.30 M KCl (Fig. 4), the titrations of both acid-*R*-paramyosin and β-paramyosin starting in base are significantly different. A previous report had suggested that paramyosin was completely stable between pH 2 and 12 (Lowey, 1965). Nevertheless, the unexpected shape of our high ionic strength titration curves starting in base suggested that the proteins had been modified by exposure to pH 12 for a period of time. Gross changes in conformation or degradation were ruled out when it was shown that dialysis against 0.30 M KCl at pH 12 for 24 h did not affect the molecular weight, molecular length, or calculated percent α-helicity of acid-*R*-paramyosin, α-*R*-paramyosin, or β-paramyosin (Cooley [1978]). It was found, however, that alkali treatment leads to partial dephosphorylation of the paramyosin molecule (Cooley et al., 1979). It appears, then, that the differences between the titrations starting in acid and the titrations starting in base are caused by differences in the extent of phosphorylation of the molecule. Cooley et al. (1979) showed that dephosphorylation by alkali treatment resulted in an increase in the solubility of the different types of paramyosin between pH 6 and 8 at an ionic strength of 0.30 M. This suggests that the phosphate groups are involved in intermolecular interactions under these conditions. Thus, by changing the intermolecular interactions, a partial dephosphorylation by alkali treatment could also result in the changes noted in the titration curves.

The similarity of the curve for β-paramyosin reported by Riddiford and Scheraga (Fig. 4) to our curves which started in acid is probably due to the fact that their samples remained phosphorylated during their whole titration procedure; that is, the extent of phosphorylation of their samples was probably similar to that of our phosphorylated samples. Although they exposed their sample to pH 12 during the titration, the length of exposure was probably very short, since their entire titration took only 3 h. The extent of dephosphorylation of other proteins has been shown to be time-dependent, requiring ~24 h for 90% dephosphorylation (Adelstein et al., 1973).

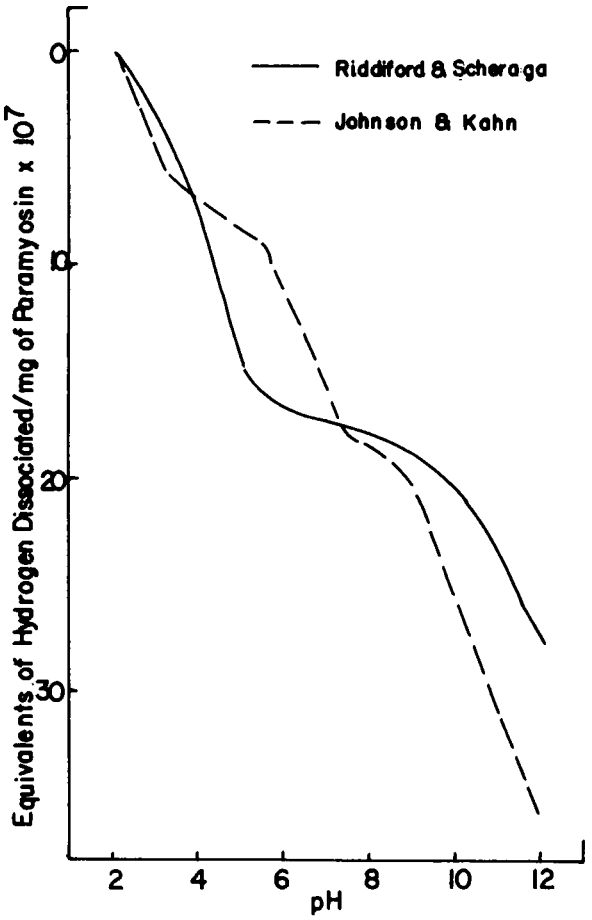

FIGURE 4 Titration curves of paramyosin previously reported in the literature by Riddiford and Scheraga (1962) and Johnson and Kahn (1959) in 0.3 M KCl.

Fig. 4 shows that the titration curve of β- and γ-paramyosin by Johnson and Kahn (1959) exhibited several regions where many groups titrated over a small range of pH similar to the region pH 9–8 we noted in our titrations which started in base (Fig. 3). However, the number and location of the regions in the curve of Johnson and Kahn differed from that seen in our curves. This may be due to a difference in the extent of dephosphorylation of our samples and theirs. The extent of phosphorylation of a sample seems to be highly dependent on several factors during the isolation and purification of the sample (Cooley et al., 1979), as well as length of exposure to alkaline solutions.

Titrations in 1 mM K^+

Unlike the pH titrations of paramyosin in 0.30 M KCl, the titrations in 1 mM KCl starting in acid superposed those starting in base. This reversibility exists even though dialysis against 1 mM KOH for 24 h leads to dephosphorylation similar to that observed in 0.3 M KCl, pH 12.

Preliminary experiments (Cooley, 1978) have shown that dialysis of α-*R*-paramyosin against 0.89 mM KOH for 24 h at 4°C decreases the number of phosphates per molecule from 3.0 ± 0.7 to 1.7 ± 0.05. Similar treatment of β-paramyosin results in a decrease from 4.9 ± 0.6 to 1.3 ± 0.7. This suggests that the phosphate groups do not have the same pronounced effect on electrostatic interactions and intermolecular interactions at low ionic strength as they seem to have in 0.3 M KCl. Similar conclusions may also be drawn from previously reported solubility data (Cooley et al., 1979). In 0.3 M KCl, dephosphorylation by alkaline treatment resulted in an increase in solubility of acid-*R*-paramyosin, α-*R*-paramyosin, and β-paramyosin between pH 6 and 8. However, as the ionic strength decreased at pH 7.00, the difference between the solubility of phosphorylated and partially dephosphorylated paramyosin decreased. At any ionic strength below 0.05 M, both phosphorylated and partially dephosphorylated paramyosin are completely soluble.

Also unlike the pH titrations of paramyosin in 0.30 M KCl, there were significant differences between the titrations of the native molecule and the partly degraded molecule in 1 mM K^+. As shown above in Table I, more groups titrated on acid-*R*-paramyosin than on β-paramyosin between pH 3 and 10, between pH 3.2 and 3.3, and in the pH region in which the molecules are insoluble.

The titration curve obtained here can be directly compared with the results of DeLaney and Krause (1976), which were also obtained at ~1 mM ionic strength. At pH 10 they have shown that both native and β-paramyosin are monomeric and exhibit the same transient electric birefringence properties; we have shown that the charges on the molecules are identical. At pH 3.2, DeLaney and Krause reported that the molecules are also monomeric but that their properties are significantly different. Their data suggests that α-*R*-paramyosin is more highly charged than β-paramyosin; our data support that conclusion. As the pH is increased from pH 3.2 to 3.3, both the specific Kerr constant and the birefringence relaxation time of α-*R*-paramyosin decrease until they are the same as the values reported for β-paramyosin. DeLaney and Krause suggested that this is due to the titration of extra groups on the extra piece on α-*R*-paramyosin. Our data support that suggestion, because α-*R*-paramyosin and acid-*R*-paramyosin have more groups titrating between pH 3.2 and 3.3 than β-paramyosin.

As noted in the Introduction, Krause and DeLaney (1977) found a shift in the aggregation behavior of native paramyosin with respect to β-paramyosin by one pH unit between pH 6 and 10 at 1 mM ionic strength. They suggested that this shift might be due to a shift in the titration behavior of the two samples by 1 pH unit. Inspection of Fig. 2 and Table I shows this to be unlikely. As shown in Table I, native paramyosin has a charge of −88 at its pH of maximum aggregation, 9.0, whereas β-paramyosin has a charge of only −66 at its pH of maximum aggregation, 7.8. This suggests that the net charge on the molecule does not by itself control the degree of aggregation.

Krause and DeLaney (1977) observed the presence of very large, side-by-side aggregates of both α-*R*-paramyosin and β-paramyosin which probably contain at least 100 monomers at pH 6. As the pH was raised above pH 6.6, the aggregates of β-paramyosin disappeared, whereas the aggregates of α-*R*-paramyosin remained until the pH was raised to 7.3. Contrary to the suggestions of Krause and DeLaney, the extended pH region of these very large aggregates of native paramyosin does not appear to be due to a shift in the titration of native paramyosin with respect to β-paramyosin (Fig. 2), nor is the charge on the two molecules the same in this

region. Between pH 6.0 and 7.3, the charge on acid-*R*-paramyosin varies from −8 to −63. Between pH 6.0 and 6.5 the charge on β-paramyosin varies from −3 to −14.

CONCLUSIONS

(*a*) Under conditions where both monomeric native paramyosin and β-paramyosin have the same physical properties (pH 10 and 1 mM ionic strength), both molecules have identical charge. Under conditions where the monomeric molecules exhibit different physical properties (pH 3.2 and 1 mM ionic strength), the calculated charges on the molecules are different. This suggests that the net charge on the molecules is important in determining the difference between the physical properties of the monomeric molecules.

(*b*) A change in the pH region in which native paramyosin aggregates at 1 mM ionic strength with respect to the aggregation of β-paramyosin does not seem to be due to simply a shift in the pH region in which certain groups titrate. Furthermore, the degree of aggregation and solubility of paramyosin at 1 mM ionic strength cannot be directly correlated with net charge. In addition, removal of one or more highly charged groups, the alkali sensitive phosphate groups, did not significantly modify the titration behavior at 1 mM ionic strength. Thus, the net charge on the molecules is not important in determining differences in aggregation behavior at 1 mM ionic strength.

(*c*) Phosphorylated and partially dephosphorylated paramyosin titrate similarly in 1 mM K^+, but very differently in 0.3 M KCl. The major differences in the titration curves occur above pH 8.

This work was supported by grants PCM 75-06456 and CHE 77-10046 from the National Science Foundation. This research was taken, in part, from a thesis by Dr. Cooley in partial fulfillment of the requirements for a Ph.D. from Rensselaer Polytechnic Institute, Troy, N.Y., 1978. A portion of the work was presented at a meeting of the Biophysical Society, Washington, D.C., 1978.

Received for publication 18 July 1979 and in revised form 14 April 1980.

REFERENCES

ADELSTEIN, R. S., M. A. CONTI, and W. ANDERSON. 1973. Phosphorylation of human platelet myosin. *Proc Natl. Acad. Sci. U.S.A.* **70**:3115.

COOLEY, L. B. 1978. The effect of pH on the physical properties of molluscan paramyosin. Ph.D. Dissertation, Rensselaer Polytechnic Institute, Troy, N.Y.

COOLEY, L. B., W. H. JOHNSON, and S. KRAUSE. 1979. Phosphorylation of paramyosin and its possible role in the catch mechanism. *J. Biol. Chem.* **254**:2195.

DELANEY, D. E., and S. KRAUSE. 1976. Properties of monomeric paramyosin using transient electric birefringence. *Macromolecules.* **9**:455.

EDWARDS, H. H., W. H. JOHNSON, and J. P. MERRICK. 1977. Comparison of solubility properties of α-paramyosin, β-paramyosin, and acid-extracted paramyosin. *Biochemistry.* **16**:2255.

GAFFIN, S. L. 1967. Properties of paramyosin in distilled water and detergent solutions. Ph. D. Dissertation, Rensselaer Polytechnic Institute, Troy, N.Y.

HODGE, A. J. 1952. Type of periodic structure obtained by reconstitution of paramyosin from acid solution. *Proc. Natl. Acad. Sci. U.S.A.* **38**:850.

JOHNSON, W. H., and J. S. KAHN. 1959. Titration of the protein paramyosin. *Science (Wash. D.C.)* **130**:1190.

JOHNSON, W. H., J. S. KAHN, and A. G. SZENT-GYÖRGYI. 1959. Paramyosin and contraction of "catch muscles." *Science (Wash. D.C.).* **130**:160.

KRAUSE, S., and D. E. DELANEY. 1977. A study of paramoysin aggregation using transient electric birefringence techniques. *Biopolymers.* **16**:1167.

LOWEY, S. 1965. Comparative study of the α-helical muscle proteins. *J. Biol. Chem.* **210**:2421.

MELSON, G. L., and R. W. COWGILL. 1976. Comparison of the muscle protein paramyosin from different molluscan species. *Comp. Biochem. Physiol. B. Comp. Biochem.* **55**:503.

MERRICK, J. P. 1975. Solubility and aggregation characteristics of molluscan paramyosin. Ph. D. Dissertation, Rensselaer Polytechnic Institute, Troy, N.Y.

MERRICK, J. P., and W. H. JOHNSON. 1977. Solubility properties of alpha-reduced paramyosin. *Biochemistry.* **16**:2260.

RIDDIFORD, L. M., and H. A. SCHERAGA. 1962. Structural studies of paramyosin. I. Hydrogen ion equilibria. *Biochemistry.* **1**:95.

RUEGG, J. C. 1961. Proteins associated with contraction in lamellibranch "catch" muscles. *Proc. Roy. Soc. Lond. B. Biol. Sci.* **154**:209.

RUEGG, J. C. 1971 Experiments on glycerinated muscle fibers. *Physiol. Rev.* **51**:201.

STAFFORD, W. F. 1973. Physical and chemical properties of paramyosin from *Mercenaria mercenaria.* Ph.D Dissertation, University of Connecticut, Storrs, Conn.

STAFFORD, W. F., and D. A. YPHANTIS. 1972. Existence and inhibition of hydrolytic enzymes attacking paramyosin in myofibrillar extracts of *Mercenaria mercenaria. Biochem. Biophys. Res. Commun.* **49**:848.

STEINHARDT, J., and M. HARRIS. 1940. Combination of wool protein with acid and base: hydrochloric acid and potassium hydroxide. *J. Res. Nat. Bur. Stand.* **24**:355.

SZENT-GYÖRGYI, A. G. 1960. Proteins of the myofibril. *In* The Structure and Function of Muscle. G. H. Bournes, editor. Academic Press, Inc., N.Y. **2**:1.

TANFORD, C. 1962. The interpretation of hydrogen ion titration curves of proteins. *Adv. Protein Chem.* **17**:69.

YEUNG, A. T., and R. W. COWGILL. 1976. Structural difference between α-paramyosin and β-paramyosin. *Biochemistry.* **15**:4654.