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# Slow Lowering of pH Induces Gel Formation of Myosin

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*ABSTRACT*

*It has been found that solutions of myosin (10 mg/ml) form gels at 5 °C if the pH is decreased slowly, by dialysis, to a value in the region of 2·5 to 5·5. Gel strength displays strong dependence on final pH, having a maximum at about pH 4·5. Salt (KCl) concentration was found to affect gel strength positively and linearly. Differential scanning calorimetry revealed that the myosin of pH-induced gels absorbed no thermal energy when heated, implicating acid-induced denaturation as the basis of gel formation. By comparison with heat-induced gelation of myosin and from the fact that low pH is conducive to filament formation, it is suggested that filaments may also be involved in the gelation process.*

## INTRODUCTION

Gelation of myosin solutions/suspensions is usually induced by heating, both in the case of laboratory experiments and as part of production procedures in meat processing plants. Fundamental aspects of the heat-induced gel formation of myosin have been investigated extensively over the past few years. The following are among the conclusions reached. Gelation in 0·4–0·6M KCl proceeds by initial aggregation of the myosin 'heads' (disulphide bonds form) and subsequent network formation upon unfolding of the tail (rod) portion of the molecule (Samejima *et al.*, 1981).

Gel strength is strongly dependent on pH, having a pronounced maximum at pH 6·0 (0·6M KCl), while the salt concentration in the range 0·4–1·0M (pH 6·0) makes no difference (Ishioroshi *et al.*, 1979). In 0·1–0·2(0·3)M KCl interfilamental aggregation of myosin heads on filament surfaces is responsible for gel formation (Ishioroshi *et al.*, 1983) and gel strength is positively correlated with salt concentration (Ishioroshi *et al.*, 1979).

Recently gels were reported to form upon addition of certain salts to myosin solutions (Nakayama *et al.*, 1983). Local 'melting' of the helical protein structure was suggested to lead to gelation.

Myosin has an isoelectric point of 5·4 (Szent-Györgyi, 1951) and studies of changes in myosin conformation (stability) induced by variations in pH have generally been limited to higher pH values. Reported findings show either that thermal stability decreases when pH is lowered to 5·5 (Goodno *et al.*, 1976; Wright & Wilding, 1984), or remains essentially unaffected (Samejima *et al.*, 1983). In a study by Ishioroshi *et al.* (1981) it was found that heat-induced development of turbidity in dilute solutions of heavy meromyosin (HMM) shows strong dependence on pH, occurring at a much lower temperature at pH 5·0 than at pH 6·0.

In this paper we report the formation of myosin gels in the cold (5 °C) by simple, slow lowering of solution pH, to 2·5–5·5, in a dialysis procedure. Further studies of the gels, using scanning electron microscopy, will be reported elsewhere (Hermansson *et al.*, 1985).

## MATERIALS AND METHODS

Myosin was isolated from bovine *M. semimembranosus*, essentially in accordance with the methods of Offer *et al.* (1973) and Nauss *et al.* (1969), at 5 °C. In the first extraction step 0·3 litre of a modified Guba–Straub solution (0·3M KCl, 0·1M $KH_2PO_4$, 0·05M $K_2HPO_4$, 4mM Na-pyrophosphate, 1 mM EDTA, pH 6·5) was added per 100 g of minced meat and stirred for 10 min. Then 0·3 litre $H_2O$ per 100 g meat was slowly added while stirring and the suspension was centrifuged at 3000 g for 20 min (Beckman JA 14-rotor, 5000 rpm). The supernatant was filtered, 6·5 volumes of distilled water containing 1 mM EDTA were added and a crude myosin sediment settled within 2 h. The sediment was centrifuged at 10 000 g for 20 min (JA 14-rotor, 8000 rpm) and resuspended in 0·1 litre KCl solution (3·0M KCl, 25 mM TES buffer reagent (SIGMA T1375), pH 7·0) per 100 g sediment; this suspension was gently stirred overnight.

The concentration of KCl was then reduced to 0·6M by adding 1·5 volumes of distilled water, and $MgCl_2$ and Na-pyrophosphate were added to a final concentration of 5 mM each. The preparation was stirred vigorously for 10 min, centrifuged at 110 000 g for 60 min (Beckman 45 Ti-rotor, 30 000 rpm) and the myosin in the supernatant was isolated by fractionated ammonium sulphate precipitation between 35 and 48% saturation: (i) saturated ammonium sulphate was added in the course of 2 min, while stirring, to 35% saturation; (ii) further 10 min of stirring; (iii) centrifugation at 16 000 g for 15 min (JA 14-rotor, 10 000 rpm); (iv) addition of saturated ammonium sulphate to the supernatant to 48% saturation; (v) another 10 min of stirring; (vi) centrifugation at 10 000 rpm for 15 min. The sediment was dissolved by adding, while stirring, 0·15 litre of phosphate buffer (0·02M $NaH_2PO_4/Na_2HPO_4$, pH 7·5), containing 1·0M KCl, per 100 g sediment and dialysed against the same buffer (2 × 1·0 litre, 24 h) prior to centrifugation at 50 000 g for 30 min (Ja 20-rotor, 20 000 rpm). Finally, the supernatant was filtered to give a clear myosin solution.

The preparation contained ≥95% myosin as judged from sodium dodecyl sulphate electrophoresis in polyacrylamide gels. The stock solution for all gelation experiments was 10 mg/ml, pH 7·5, 0·6M KCl in 0·02M phosphate buffer. All chemicals used were of analytical quality.

Gels were formed in plastic ('Perspex') cylinders of 24 mm inner diameter and 13 mm height (Smidsrød *et al.*, 1972). Flat pieces of dialysis tubing were stretched over the ends of the cylinders and held in place with rubber bands; it required special attention to avoid air bubbles when closing the cylinder with the second piece of dialysis tubing. Dialysis proceeded at about 5°C (cold laboratory) against a stirred 0·01M citric acid/0·02M phosphate buffer of varying pH and KCl concentration. The ratio of myosin solution volume to dialysate buffer volume was about 1:80 and the buffer was changed once in the course of about 44 h. In a two-step gel formation procedure gels were first formed as described above and subsequently subjected to another dialysis treatment to attain a different, final ionic strength.

Differential scanning calorimetry (DSC) was carried out in a Perkin-Elmer DSC II instrument, using a scanning rate of 10°C/min and a sensitivity of 0·5 mcal/s. Myosin gels were compressed by centrifugation and samples of the moist pellets were placed in 75 μl stainless steel sample pans. The non-gelled myosin sample at pH 5·5 (giving Fig. 1a) was prepared by suspending myosin, which had been precipitated by ammonium sulphate, in dialysis buffer (1:2, w:w) shortly before

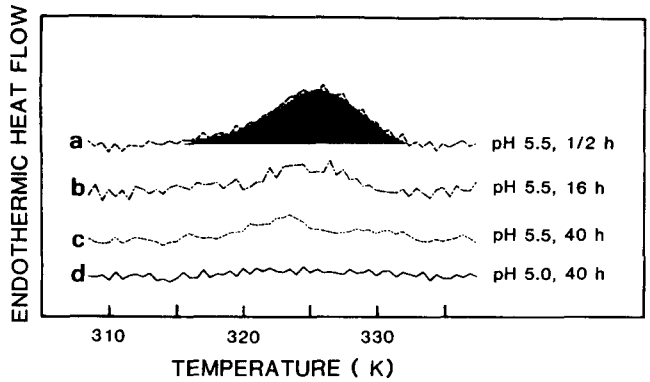


**Fig. 1.** Thermograms, obtained by differential scanning calorimetry, depicting the effect of exposure to low pH (0·01 M citric acid/0·02 M phosphate buffer, 0·6 M KCl) on myosin at 5 °C. The amount of protein subjected to calorimetry was about the same in all four cases. The area under the peak (see Fig. 1a) is proportional to the apparent enthalpy of heat denaturation of the protein.

calorimetric measurement. The protein concentration of the DSC samples was estimated by drying at 105 °C overnight.

Measurements of gel strength were carried out at room temperature, about 22 °C, in an Instron Universal Testing Machine by indentation at 2·5 mm/min crosshead speed, plunger diameter 5 mm, after removal of the dialysis membrane at the top of the cylinder. The initial slope of the force–deformation curve was taken as a measure of gel strength. The measurements were corrected for the buoyancy of the plunger when 'penetrating' an unheated protein solution. Measured gel strengths were normalized to relative values since absolute gel strengths are affected by differences in the meat raw material, minor variations in the isolation and storage of myosin preparations and uncertainties in protein concentrations. There was virtually no variation among the preparations used in the relative dependence of gel strength on pH and salt concentration.

The pH of the gels formed was verified by direct measurements in the gels after Instron measurements.

The concentration of KCl in the liquid phase of the gels was checked by conductivity measurements of the clear supernatant obtained by centrifuging gels for 30 min at 20 000 rpm in a Beckman JA-20 rotor.

Protein concentrations were calculated from optical density at 280 nm, using $E^{1\%}_{280} = 5{\cdot}70$ (Highsmith *et al.*, 1979).

The turbidity of diluted myosin suspensions (1 mg/ml) after the usual dialysis procedure was estimated as their optical densities at 340 nm.

## RESULTS

The thermograms of Fig. 1a–c show that there is a dramatic decrease in apparent enthalpy of heat denaturation (proportional to peak area, *cf.* Fig. 1a) with time at pH 5·5. Myosin treated at 5·0 is seen to absorb no heat at all upon heating in the calorimeter (Fig. 1d). It was deemed superfluous to run samples gelled at lower pH values.

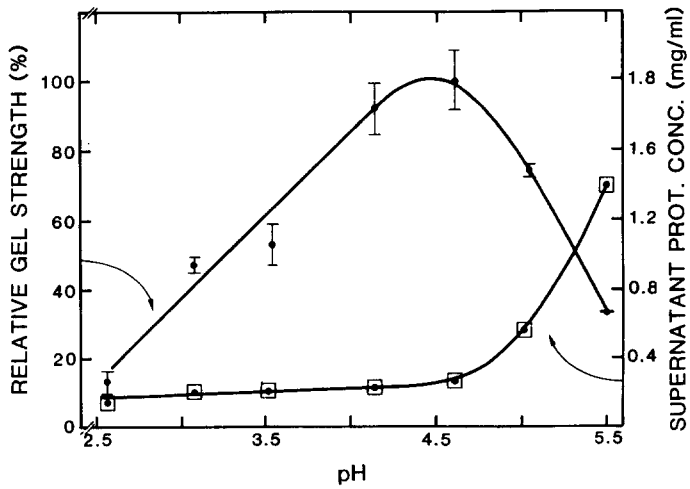


**Fig. 2.** Effect of final pH on (i) the gel strength and (ii) the protein concentration of the liquid phase of myosin gels induced by slow lowering of pH from 7·5 at 5 °C in 0·02M phosphate buffer, 0·6M KCl; 10 mg protein per millilitre. The gels had reached pH equilibrium with the dialysate. Bars indicate ± standard error of the mean.

Figure 2 shows the dependence of gel strength on pH for myosin gels at 0·6M KCl. It is seen that gel strength passes through a pronounced maximum at about pH 4·5. Furthermore, it should be noted that gels retain considerable amounts of protein in the liquid phase at a final pH $\geq$ 5·0.

Figure 3 reveals that there is a positive, apparently linear, relationship between gel strength and salt concentration. The results given were derived from two different experimental procedures. The dialysis treatment was either carried out as a one-step procedure—i.e. dialysis against a buffer of pH 4·5 and various KCl concentrations—or as a two-step procedure in which pH 4·5 was first established and then, in a second

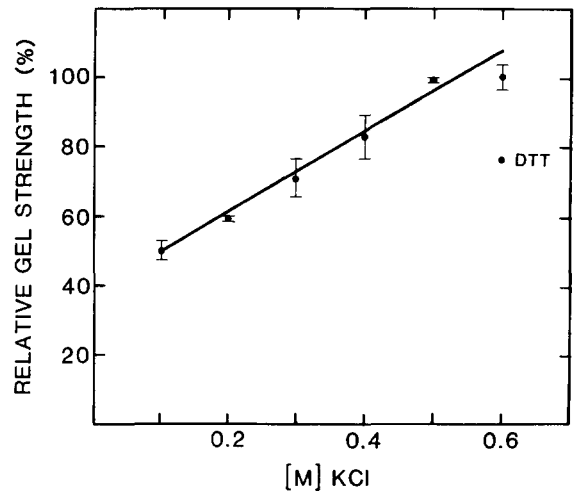


**Fig. 3.** Effect of final salt concentration (initial concentration, 0·6M KCl) on the strength of myosin gels induced by slow lowering of pH to 4·5. Effect of DTT determined at 1 mM concentration. Other conditions as for Fig. 2. Bars indicate ± standard error of the mean.

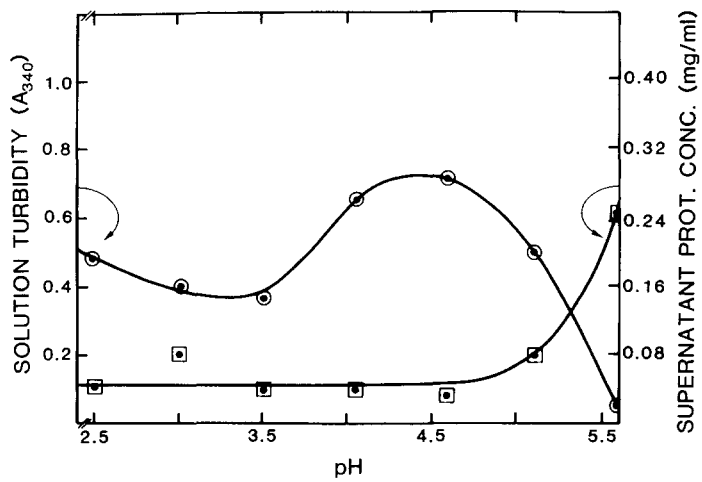


**Fig. 4.** Effect of final pH on (i) solution turbidity and (ii) supernatant protein concentration when treating a dilute myosin solution (1 mg/ml) in the same way that gels were made; *cf.* Fig. 2.

dialysis step, the KCl concentration changed to the values indicated. The two procedures yielded indistinguishable results, the combination of which is given in the Figure.

Addition of dithiothreitol (DTT), which prevents the formation of disulphide bonds, causes a significant ($p < 0{\cdot}01$) decrease in gel strength (Fig. 3). It is evident from the Figure, however, that DTT merely has a certain deleterious effect; it clearly does not totally prevent gel formation.

Figure 4 gives the results of a series of dialysis treatments in which a concentration of only 1 mg/ml of myosin was used. Due to the low concentration, gels were not formed but turbidity developed to varying extents, as indicated. It should be noted that the curve for the supernatant protein concentration takes a course quite analogous to the corresponding one in Fig. 2. Similarly, at pH 3·5 and above, solution turbidity displays a dependence on pH which essentially parallels that of gel strength (Fig. 2). At lower pH values, however, turbidity increases (Fig. 4) while gel strength continues to decrease (Fig. 2). Turbidity measurements at 660 nm verified the turbidity curve given (obtained at 340 nm).

## DISCUSSION

Gelation of protein solutions of sufficient concentration, indeed of any solution of macromolecules displaying gel-forming ability, arises either from a change pertaining to the macromolecule or from a change in its surrounding medium, or both. If the protein in question is essentially native, as the myosin of our stock solution, a conformational change needs to be induced.

The results of Fig. 1 demonstrate that there is a conformational change involved in the acid-induced gelation of myosin. While Wright & Wilding (1984) found that myosin denaturation enthalpy was decreased when pH was lowered to 5·5, it is seen in Fig. 1 that prolonged exposure to low pH values eliminated the protein's absorption of heat in the calorimeter (Fig. 1d). It is concluded that acid-induced denaturation had taken place prior to calorimetry, making gel formation in the cold possible.

Some aspects of acid-induced gelation may be understood from comparisons with gel formation by heat at a neutral pH value. Initially, the increase in gel strength observed with decreasing pH values (to pH 4·5, Fig. 2) corresponds to the observation: the higher the temperature, the

stronger the heat-induced gel (up to about 60 °C; Ishioroshi *et al.*, 1979). In both cases stronger denaturing conditions (lower pH or higher temperature) lead to stronger gels. One direct reason for relatively weak gels at pH ≥ 5·0 is evident from Fig. 2: the higher pH values imply that higher amounts of myosin remain dissolved in the liquid phase of the gel; i.e. the gel network is made from less protein in this pH region. This may be due to incomplete protein denaturation, cf. Fig. 1c.

The strength of heat-induced gels reaches a plateau when gel formation takes place at a sufficiently high temperature (≥60 °C; Ishioroshi *et al.*, 1979). The situation with acid-induced gels is more complex. The lowering of pH from the isoelectric point, pH 5·4, also increases the positive charge on the myosin molecules and thereby the repulsion between them. Presumably gel strength decreases below pH 4–4·5 because the gel network formed initially remains sufficiently dynamic to allow increased intermolecular repulsion, on further pH decrease, to have a deleterious effect on the strength of the network. It should be kept in mind, however, that, in the case of heat-induced gelation of globular proteins at high (≥0·3M) ionic strength, the opposite effect is seen: decreasing pH, when below pI, increases gel strength (Egelandsdal, 1980).

Gelation—that is, the formation of a continuous network throughout a solution—cannot be effected at concentrations below a certain limit. Instead small, aggregated particles are formed, making the solution turbid. The turbidity data of Fig. 4 show obvious covariation with the results for gel strength (Fig. 2) at pH > 3·5. The reasons for the difference in pH dependence at low pH (2·5–3·5) are not clear.

The effect of decreasing the salt concentration at pH 4·5 (Fig. 3) is a little surprising in view of the complex relationship found for heat-induced myosin gels (Ishioroshi *et al.*, 1979). However, a qualitative understanding of the apparent linear relationship may be attempted by considering the two-step experimental procedure: in the initial equilibration of the 0·6 M KCl solutions to pH 4·5 gels are formed—Fig. 2. Subsequent lowering of the ionic strength of the liquid phase probably has an effect similar to a decrease in pH (discussed above) by decreasing the 'shielding' of charges on the protein molecules, thereby increasing the electrostatic repulsion between them.

Since the diffusion coefficient of $H_3O^+$ is only about twice as high as that of $Na^+$, there can be no doubt that the series of one-step dialysis experiments implied widely varied salt concentrations within the dialysis bags during gel formation. Nonetheless, the same dependence of gel hardness on final salt concentration was found as with the two-step

procedure. These data suggest, therefore, that the gel-forming process is highly dynamic, giving *final* pH values and salt concentrations decisive importance for gel strength.

Addition of DTT decreases gel strength (Fig. 3), suggesting that the formation of disulphide bonds is involved in network formation. Again acid-induced gelation may be analogous to heat-induced gelation by including an element of head-to-head aggregation involving disulphide bond formation (Samejima *et al.*, 1981), even at these low pH values.

In the study by Ishioroshi *et al.* (1979), working at pH 6·0, heat-induced gels were made either from essentially monomeric myosin ($\geq$0·4M KCl) or from filamentous myosin ($\leq$0·2M KCl). As seen from their work, using stock myosin stored in 0·6M KCl, there is an increase in gel strength when the salt concentration is raised from 0·1M to 0·2M. Hence, the positive correlation seen in Fig. 3 raises the question of a possible rôle of filaments in the formation of acid-induced gels.

Two observations make filament formation conceivable at the high ionic strength in question (0·6M KCl). Working at an intermediate ionic strength (0·3M KCl), Kaminer & Bell (1966) found a sharp increase in filament length when decreasing the pH to 6·0. Sanger (1971) reported similar findings in addition to showing that a dialysis procedure, as opposed to rapid dilution, is conducive to the growth of long filaments. Since the ability to form filaments is lost during the initial stages of myosin denaturation (Yasui *et al.*, 1966), filament formation would need to precede denaturation. The filamentous character of the gels formed at pH 4·0 has recently been confirmed by electron microscopy (Hermansson *et al.*, 1985). Nonetheless, extensive further work is needed before conclusions can be reached with regard to the specific mechanisms involved in acid-induced gelation of myosin.

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