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Infrared Spectroscopic Studies on the Conformation of Myosin in Films†

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ABSTRACT: The infrared spectra of myosin films prepared under a variety of conditions have been studied. The precipitating conditions determine whether β structure as well as the α helix is observed. In films which have been prepared by precipitation from low ionic strength aqueous media (which approach physiological conditions) the myosin appears to be a mixture of random and α-helical conformation. A very high degree of enzymatic activity is retained by the myosin in the dried films indicating no major irreversible changes on precipitation and dehydration under N_2. Lower activity is observed from films which have been prepared by lyophilizing myosin from solution at high ionic strength. In films which have been prepared by precipitation in aqueous media at low pH, the infrared spectra indicated a significant proportion of β structure has been induced by the pH change. The observed conformations also depend on the precipitating media when organic solvents were used to prepare films. If pure methanol or a 50:50 mixture of methanol and chloroform were used in the precipitating media, β structure was observed while with pure $CHCl_3$ there was no indication of β structure. The possible role of β structure in muscular contraction is of course purely speculative, but an extension of length on formation of β structure is known for other polymeric systems. In considering possible mechanisms of muscular contraction, the burst of hydrogen ions produced by the hydrolysis of ATP under physiological conditions could result in a localized region of low pH. The presence of β structure at low pH suggests that an $\alpha \rightarrow \beta$ conversion should also be considered in postulating possible mechanisms for movement in the flexible hinge region of myosin to allow cross-bridge movement.

Infrared absorption spectra of polypeptides and proteins have been used in the study of conformation for a number of years. There have been numerous reviews of the correlation between spectra and conformation (Elliot *et al.*, 1962; Miyazawa *et al.*, 1967; Timasheff and Gorbunoff, 1967; Susi, 1969). The amide I band (1600–1700 cm^{-1}) which is due to the C=O stretching mode, the amide II band (1500–1550 cm^{-1}) which is due to the hybridization of the N–H bending and C–N stretching modes, as well as the amide IV band (600–700 cm^{-1}) which arises from the CONH groups have been used for conformational diagnosis. Polypeptides also exhibit other strong infrared bands such as the amide A (3300 cm^{-1}) and

† Contribution from the Department of Chemistry, University of Calgary, Calgary, Alberta, Canada. *Received November 20, 1972.* Publication No. 73-03 from the Biopolymer Research Group, University of Calgary. This work was supported by the Alberta Heart Foundation, the National Research Council of Canada, and the Defence Research Board of Canada (Grant 1675-13).

the amide B (3100 cm^{-1}) which may be the Fermi resonance between the fundamental N–H stretching vibrations and the first overtone of the amide II vibration. These bands are not very sensitive to changes in chain conformation.

Theoretical band assignments have been made for the α-helical, pleated sheet (parallel and antiparallel), and unordered conformation by analysis of the position and polarization of the bands (Miyazawa, 1960; Krimm, 1962). There is good agreement between the theoretical band assignments and the bands observed in globular proteins and synthetic polypeptides of known conformation. The principal frequencies of the amide I band for the α-helical conformation (1650 cm^{-1}) and the random conformation (1656 cm^{-1}) are close and the observed bands are broad. Considerable overlap is expected with mixed α-helical and random conformations. Hence discrimination between the α-helical and a random conformation in a sample of unknown conformation is difficult. The principal amide I frequency for both the parallel and antiparallel pleated sheet is in the range of 1630–1632 cm^{-1}. This is a shift of 20 cm^{-1} toward lower frequency than for the α-helical conformation. Hence it is possible to determine whether some β structure is present even though the bands are broad and the major contribution is from the α-helical or random conformations. The presence of a shoulder near 1630 cm^{-1} and the presence of a band in the 1685- to 1700-cm^{-1} region has been used as a diagnostic tool indicating the presence of some β structure in proteins which possess more than one type of secondary structure (Timasheff and Gorbunoff, 1967). β structure in native β-lactoglobulin was first identified by infrared (ir) spectra (Timasheff and Susi, 1966). The presence of β structure in lysozyme was also suggested by the ir spectra (Hamaguchi, 1964).

Since H_2O has a strong absorption band near 1650 cm^{-1}, solution studies of proteins and polypeptides are usually carried out in D_2O. Deuteration shifts the amide bands toward lower frequencies (Suzuki *et al.*, 1966) but does not alter the conformation dependent differences in the principal amide frequencies.

Optical rotatory dispersion (ORD) (Tonomura *et al.*, 1963) and circular dichroism (CD) (Mommaerts, 1966; Oikawa *et al.*, 1968) measurements of myosin, the major contractile protein, have established that the protein in solution contains about 50–60% of the α-helical conformation. No other type of secondary structure has been observed in myosin in solution. Numerous theories of muscle contraction have postulated as an intrinsic part of the mechanism for muscular contraction, a conformational change in myosin (Flory, 1956; Prior, 1950). Harrington (1972) postulated a helix–coil transition in myosin as part of the contractile mechanism. No macroscopic evidence has been reported for such a conformational change. In view of the high helical content of the rod portion of the myosin molecule a localized helix-to-coil conformational change could easily not be detectable by conventional experimental techniques. Fluorescent studies have indicated the possibility of a microscopic change in conformation at or near the active site of the molecule (Cheung and Morales, 1969). However, changes in fluorescence cannot indicate the type of change occurring in the molecule. The possibility that a localized conformational change in the myosin molecule might involve β structure has not been previously considered since the ORD–CD analysis has shown no evidence of β structure in myosin. There is no *a priori* reason to expect the presence of any β structures.

Most studies of myosin (optical rotation, circular dichroism, and fluorescence) which might indicate a conformational change are of myosin in the solution form at a high ionic strength. Under physiological conditions myosin is in the gel or precipitated form. Hence studies of myosin in a gel or precipitated form would be of value in determining the conformation of the protein and the possibility of conformational changes. However, myosin presents a problem in film preparation. Freeze-drying can be used to prepare myosin films, but the possibility of structural changes on dehydration cannot be eliminated. With synthetic polypeptides such as poly(L-lysine) (Blout and Lenormant, 1957; Elliot *et al.*, 1957) and poly(L-glutamic acid) (Lenormant *et al.*, 1958), the observed conformation (α, β, and/or random) depends on the humidity or water content of the films. The usual method of preparing films from synthetic polypeptides and proteins is by evaporation from a nonaqueous solvent. This method also cannot be applied to myosin since myosin is insoluble in most common nonaqueous solvents. Evaporation from aqueous solution has also been used to prepare films, but myosin films prepared in this manner may not be identical with myosin films prepared under physiological conditions. We have devised a method of preparing films from myosin which takes advantage of myosin's insolubility at low ionic strength or in organic solvents, and have studied the infrared spectra of myosin films prepared under various conditions including those which closely approach the expected physiological environment.

Materials and Methods

The dorsal muscles of New Zealand white rabbits were used for all preparations. Myosin was prepared by the method of Tonomura *et al.* (1966) and chromatographed with DEAE-Sephadex as suggested by Baril *et al.* (1966). Double-distilled water was used throughout and all solutions were treated with Chelex-100 resin to remove divalent ions. All chemicals were of reagent grade or better. Myosin was stored at a concentration of 4.6–6.0 mg/ml in 0.6 M KCl at pH 7.4.

The purity of the myosin preparations was determined after decomposition by dodecyl sulfate and 8 M urea by electrophoresis in polyacrylamide gels by the method of Weber and Osborn (1969).

Films of myosin were prepared in the following manner. The myosin solution was usually concentrated to between 6 and 12 mg per ml. A wet film was made in a thin Teflon O ring by dipping the Teflon into the protein solution. The Teflon holder containing the wet film was floated on top of the precipitating medium (H_2O, dilute salt, or organic solvents), and allowed to remain for various periods ranging from 15 min to overnight. (Conditions are specified in the figures.) The precipitated film in the Teflon holder was carefully removed from the precipitating medium and dried. Various methods of drying were tried. The most satisfactory method to prepare films in which the enzymatic activity of the protein was retained was in a closed container under N_2 at 4°. For faster drying a slow stream of N_2 was passed over the film. The film was removed from the Teflon holder and placed between KBr plates which were used as the ir cell windows. The infrared spectra were obtained with a Perkin-Elmer spectrophotometer Model 621.

The enzymatic activity of the myosin was measured in 0.6 M KCl, 10 mM $CaCl_2$, 1.0 mM ATP, and 0.02 M Tris buffer at pH 7.4. The modified Fiske-Subbarow phosphate determination proposed by Lecocq and Inesi (1966) was used in these measurements. Four aliquots were taken during the 10-min period after ATP addition. There was a linear rate of production of inorganic phosphate during this time period. The con-

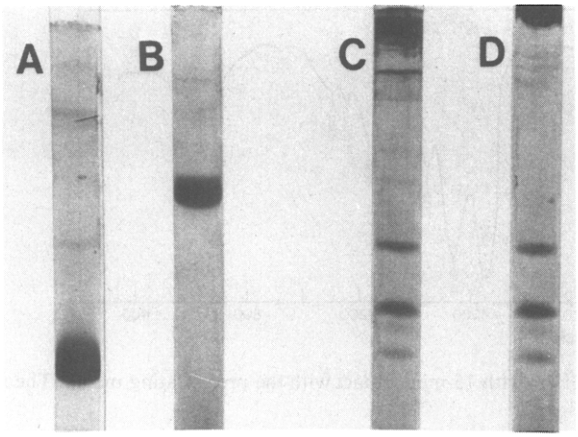

FIGURE 1: Sodium dodecyl sulfate–urea disc gel electrophoresis of myosin. A and B are standards of ribonuclease and aldolase run in the same experiment. C is the unchromatographed myosin (50-μg application) and D is the chromatographed myosin (56-μg application).

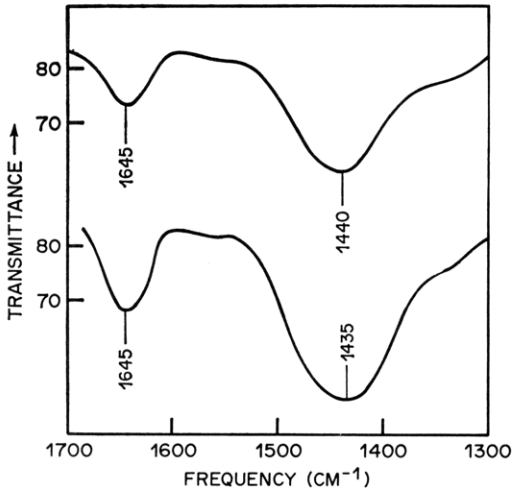

FIGURE 2: Ir spectra of myosin in D_2O. Sample A contained 4 mg/ml of protein while sample B contained 8 mg/ml of protein. The protein was reprecipitated four times in D_2O to exchange the protons in the myosin. Samples contained 0.3 M KCl in D_2O at pH 7.4. A variable path length cell (set at 0.051 mm) with CaF_2 windows was used for the spectra. A 0.3 M KCl in D_2O blank was run at the same time and substracted from the spectra.

centration of myosin in the ATPase activity determination for measurement of the standard activity of the stock myosin solution was 0.1 mg/ml. A similar concentration was used in the ATPase determination from the films but the myosin concentration is only approximate since the films were scraped into the tube for activity analysis and small losses would significantly affect activity (resulting in a lower apparent activity). The films were stirred for 15 min at 25° before ATP addition. All the myosin in the film was not soluble in the 0.6 M KCl as some particles were observed after the ATPase determination. This may be due to denaturation of part of the myosin but could be due to the slow rate of dissolution of myosin. Hence, the value obtained for activity of myosin extracted from films is a minimum value as all these factors would tend to lower the apparent rate.

Results

The sodium dodecyl sulfate–8 M urea disc gel electrophoresis results are shown in Figure 1. Results are very similar to previous disc gel electrophoresis of myosin under similar conditions by Lowey and Risby (1971).

Precipitation of the myosin at pH 7 in water or in dilute salt solution (0.15 M KCl) followed by drying under N_2 at 4° did not irreversibly destroy the enzymatic activity of the myosin. The enzymatic activity of the stock myosin solution was 8 ± 0.3 μM P_i/g per sec while the myosin from dried films stored 5 days was between 5 and 6 μM P_i per g per sec. Hence, 60–75% of the activity was retained after film preparation at pH 7. There was no significant difference in the activity of the myosin from the dried films when either water or 0.15 M KCl was used in the film preparation. Only 40–50% of the activity was retained in films which were freeze-dried from 0.6 M KCl. In the freeze-dried films storage under N_2 was not necessary. Films stored at room temperature in a desiccator for 6 days had essentially the same activity as freshly dried films which were kept at 4° during the freeze-drying process.

The ir spectra of myosin at 4 and 8 mg per ml in 0.3 M KCl in D_2O are shown in Figure 2. The myosin had been reprecipitated from D_2O four times. The spectra have been corrected for the 0.3 M KCl in D_2O blank. The principal frequency of the amide I band is at 1645 cm^{-1}. This is between the values reported for globular proteins in D_2O for the unordered conformation (1643 cm^{-1}) and the α-helical conformation (1650 cm^{-1}). The amide II band in the 1500- to 1550-cm^{-1} region which is largely due to N–H bending has almost completely disappeared indicating essentially complete exchange of the N–H hydrogens to N–D. The principal band in the spectrum of myosin in D_2O is in the region 1430–1440 cm^{-1}.

The total ir spectrum of a myosin film which was precipitated from 0.15 M KCl at pH 7.0 is shown in Figure 3. The N–H stretching frequencies appear at 3290 and 3060 cm^{-1}. Additional bands in this region appear at 2960, 2930, and 2875 cm^{-1}. Bands in this region have been associated with N–H stretching, but have not been found useful for characterization of the conformation. The amide I band from films prepared at pH 7 is centered at 1655 cm^{-1} and there is a suggestion that this may be a doublet, but the broadness of the bands and the instrument noise level makes the interpretation of this small dip uncertain. The amide II band appears at 1545 cm^{-1} but does not appear symmetrical and there is the possibility of an additional band which is not resolved in the 1500-cm^{-1} region. Additional weaker bands appear at 1450, 1390, 1300, 1240, 1170, 1120 cm^{-1} and a broad band at 630–640 cm^{-1} which is probably the amide IV band. There is the suggestion of some very weak additional bands at 1070, 950, 740, and 700 cm^{-1}. In Figure 4 the ir spectra of films prepared from pure water and 0.15 M KCl at pH 7.0 are compared. This low salt concentration is comparable to physiological conditions and there is no significant difference between the films. In a noninteracting mixture of α-helical conformation plus random conformation strong amide I, II, and IV bands would be expected at 1656 (random), 1650 (α), 1546 (α), 1535 (random), 650 (random), and 520 (α) cm^{-1}. Hence at pH 7, the myosin film appears to be a mixture of random and α-helical conformation.

The infrared spectra for myosin precipitated at pH 11 are shown in Figure 5 and appear very similar to the spectra at pH 7. The effect of the concentration of myosin in the stock solution before film preparation was investigated at all pH's. No changes were observed with protein concentration. This is illustrated in Figure 5 for pH 11. Spectra are shown for myosin at 5- and at 10-mg/ml concentration in the stock solution. In

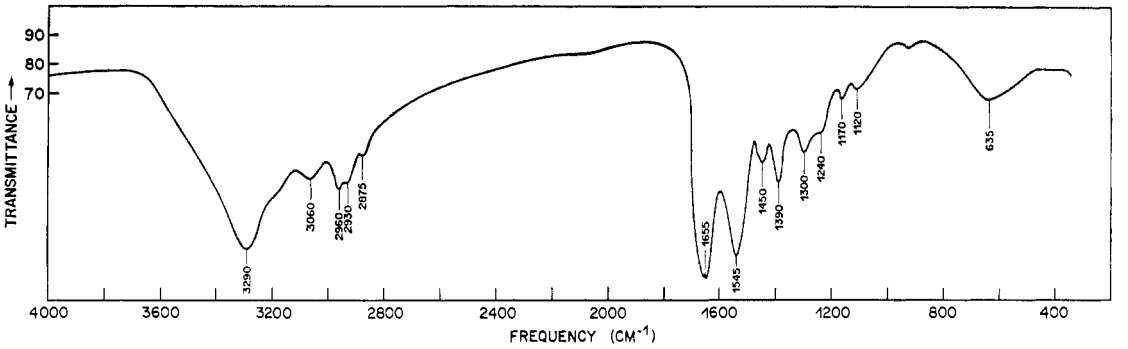

FIGURE 3: Ir spectrum of myosin precipitated as a film from 0.15 M KCl at pH 7.0 with 15-min contact with the precipitating media. The film was dried under N_2. Myosin stock solution was 8 mg/ml in 0.6 M KCl at pH 7.4.

the final spectrum the myosin film prepared from myosin at 5-mg/ml stock concentration was doubled to determine the effect of film thickness. There was no change in spectra but an increase in absorption band intensity due to the thicker film. There was no trace of any shoulder in the 1630-cm^{-1} region which would indicate the presence of β structure. Both the α helix and the random form appear to be present after base treatment. Seidel (1967) has observed that after exposure of myosin to pH 9 for 10 min at 25° only 15% of the original enzymatic activity was retained after return to pH 7. The exposure to pH 9 did not affect the optical rotation of myosin. The infrared results after exposure to pH 11 follow the pattern observed by Seidel (1967).

A wet film of myosin was prepared from a stock solution containing 10 mg/ml of protein in 0.6 M KCl at pH 7.4. This film was frozen in liquid N_2 and freeze-dried. The ir spectrum of this film is shown in Figure 6. The amide I peak is centered at 1650 cm^{-1} while the amide II appears at 1540 cm^{-1} and definitely has a shoulder. The amide IV appears at 650 cm^{-1}. In Figure 7 there also is shown a spectrum from a KBr pellet of myosin in which the myosin stock solution (10 mg/ml of protein in 0.6 M KCl at pH 7.4) was heated to 65° for 20 min and quickly frozen in liquid nitrogen. Optical rotation of myosin in 0.6 M KCl at 65° indicated a denatured protein ($\alpha_{233} = -11 \times 10^3$ at 20° and -4×10^3 at 65°). The amide I peak from the KBr pellet is centered at 1655 cm^{-1} and the amide II at 1535 cm^{-1}. There is no indication of a shoulder on the amide II peak. The amide IV appears broad and is centered around 630–640 cm^{-1}.

There is however a significant difference in the ir spectrum of myosin films prepared by precipitation at pH 1. In Figure 7 the ir spectrum of a film prepared in 0.15 M KCl at pH 1 is shown. A shoulder is observed at 1630 cm^{-1} in addition to the amide I bands observed at 1650 and 1655 cm^{-1}. This shoulder is characteristic of the amide I frequency of the β structure. A shoulder also appears in the region of 1720 cm^{-1} which may indicate the antiparallel β conformation. The ir spectrum of myosin films at pH 1 is consistent with the interpretation that random, α-helical, and β structure all coexist at this pH.

In Figure 8 the effect of protein concentration in the stock solution used for film preparation on the spectrum at pH 1 is shown. A shoulder at 1630 cm^{-1} is still visible when the pro-

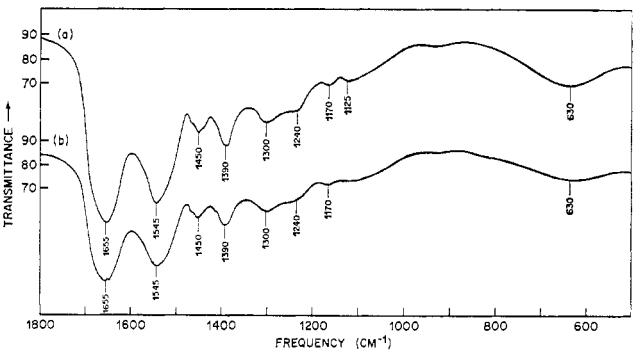

FIGURE 4: Comparison of spectra of myosin precipitated from pure water (a) and from 0.15 M KCl (b) at pH 7.0 with 15-min contact with precipitating media. Myosin stock was 10 mg/ml in 0.6 M KCl at pH 7.4. The films were air-dried. Similar spectra were observed with films which were in contact with the precipitating media for 12 hr.

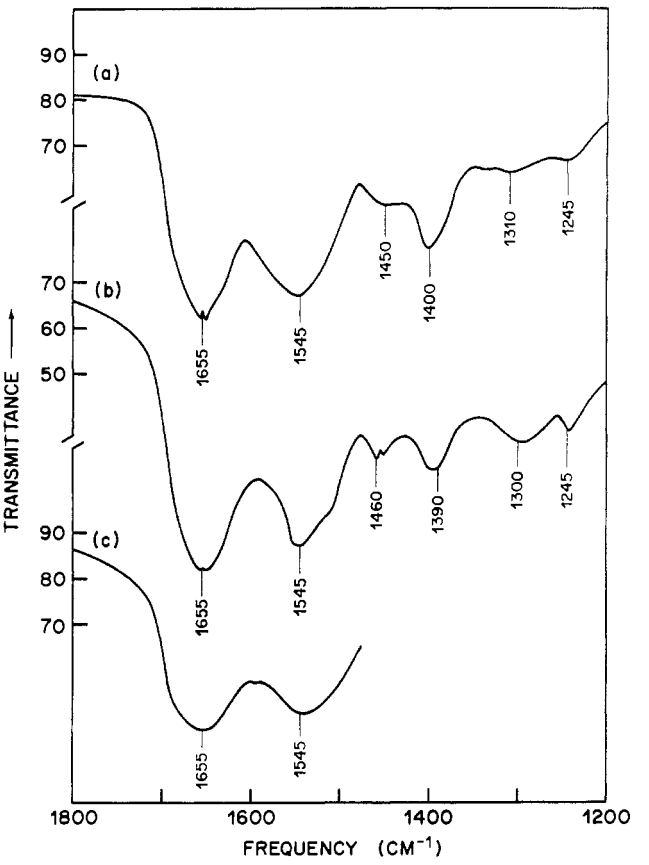

FIGURE 5: Ir spectra of films of myosin in 0.6 M KCl at pH 7.4 precipitated by contact with 0.15 M KCl at pH 11. In part a the myosin stock concentration was 10 mg/ml and the myosin was precipitated by contact with the 0.15 M KCl at pH 11 for 2 hr. In part b the myosin stock concentration was 5 mg/ml and the contact time with the 0.15 M KCl at pH 11 was 1 hr. In part c the thickness of the film in part b was doubled.

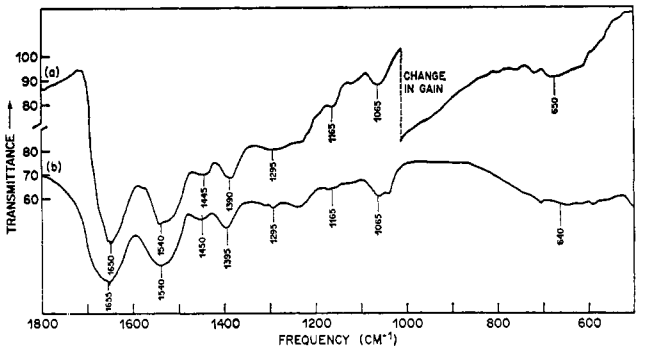

FIGURE 6: (a) The ir spectrum of a film of myosin in 0.6 M KCl at pH 7.4 which was frozen in liquid nitrogen and lyophilized. (b) Similar spectrum from a KBr pellet of a myosin solution which was heated at 65° for 20 min, quickly frozen in liquid nitrogen, and lyophilized. The myosin stock solution was 10 mg/ml of protein in 0.6 M KCl at pH 7.4.

tein concentration was decreased to 5 mg/ml. The ir spectrum of a film of unchromatographed myosin prepared at pH 1 is also shown in Figure 8. There is no change in the spectra upon chromatography.

The conformations which are observed after precipitation from nonaqueous solvents appear to depend on the specific solvent. Figure 9 compares spectra of films precipitated from pure methanol, pure chloroform, and a 50:50 mixture of these solvents. The film from $CHCl_3$ appears to be a mixture of random and α-helical conformations and the observed frequencies are very similar to films from water at pH 11 and 7. In the films from both 100% CH_3OH and the 50:50 mixture, the shoulder at 1625–1630 cm^{-1} is observed. The myosin in these films appears to be a mixed structure as in the film prepared from aqueous solution at pH 1.

The presence of the 1630-cm^{-1} shoulder characteristic of the β structure appears to depend on the initial precipitating media. In Figure 9d the spectrum of a film precipitated from an aqueous solution containing 10% methanol (15-min contact) is shown. Before drying, the wet film was placed in a 50:50 mixture of $CHCl_3$–CH_3OH for 1 hr. There is no trace of the 1630-cm^{-1} shoulder although this shoulder was readily apparent when the $CHCl_3$–CH_3OH mixture was used as the precipitating medium (Figure 9c). The spectrum shown in 9d is identical with spectra of films prepared by precipitation from an aqueous solution containing 10% methanol followed by either air-drying or drying under N_2. The absence of water from the precipitating media is also not a specific criterion for presence of β structure since films prepared from pure $CHCl_3$ did not show the 1630-cm^{-1} shoulder.

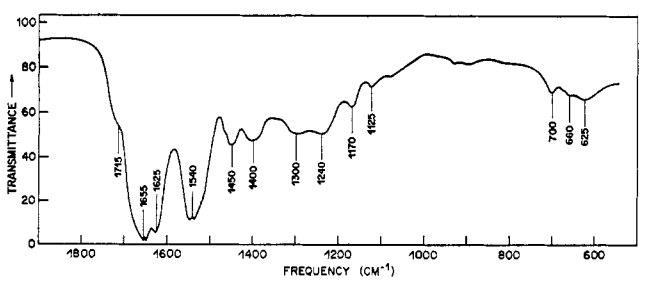

FIGURE 7: Ir spectrum of myosin which was precipitated in a film by contact for 1 hr with 0.15 M KCl at pH 1.0. The film was dried under N_2. The myosin stock solution was 10 mg/ml in 0.6 M KCl at pH 7.4.

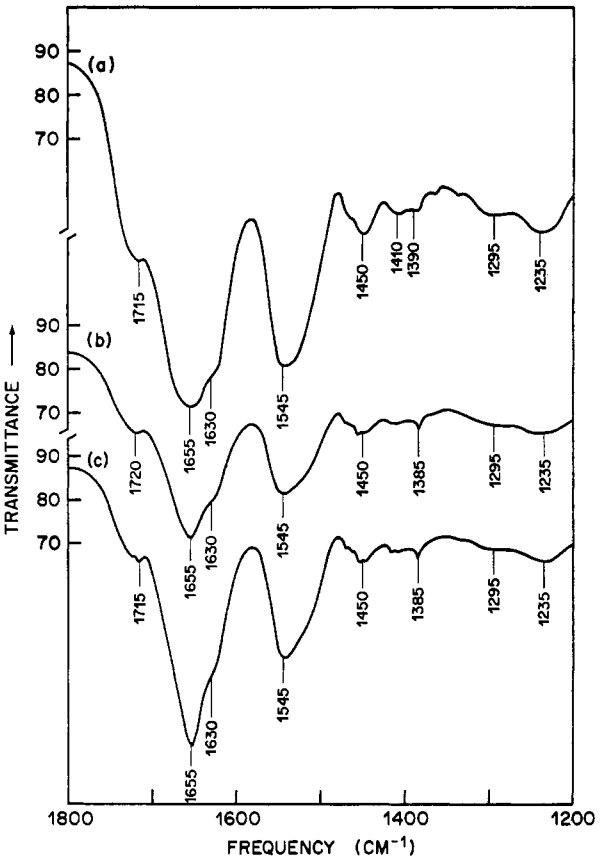

FIGURE 8: Ir spectra of myosin precipitated in a film by contact for 1 hr with 0.15 M KCl at pH 1. The film was dried under N_2. The myosin stock solution in part a was 10 mg/ml. The myosin stock solution in part b was 5 mg/ml. In part c a myosin stock solution of 5 mg/ml before chromatography was used.

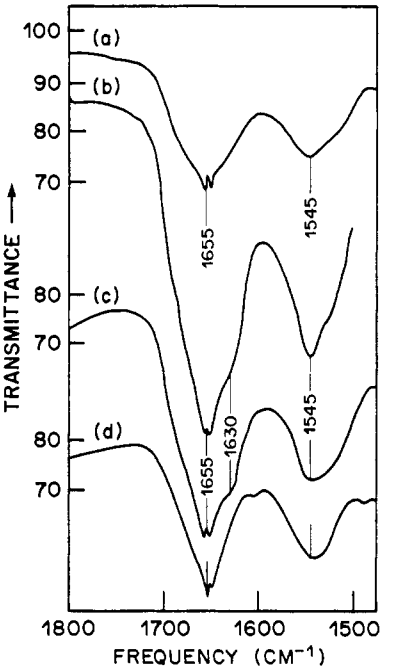

FIGURE 9: The ir spectra of myosin in the amide I and II region (a) precipitated by contact with pure chloroform, (b) precipitated by contact with pure methanol, and (c) precipitated by contact with a 50:50 mixture of chloroform–methanol. The contact time with the organic solvent was 15 min and films were subsequently air-dried. (d) Myosin precipitated by contact with an aqueous solution containing 10% methanol for 15 min followed by immersion in a 50:50 mixture of chloroform–methanol for 1 hr. The film was air-dried.

Discussion

The relatively high retention of enzymatic activity after drying of films precipitated at pH 7 indicates that there is no major irreversible change in the protein caused by drying under N_2. The apparent change in specific activity in films prepared at pH 7 could easily be due to small protein concentration differences. Hence the ir spectrum of this film at pH 7 is that of the active protein. No enzymatic activity would be expected in films prepared at pH 11 or 1 since myosin is denatured at these pH's.

The activity of the protein in films dried under N_2 is significantly higher than from films which had been freeze-dried. It is possible that there was some retention of H_2O in the films dried under N_2 and the complete dehydration by freeze-drying was responsible for the decrease in activity. A second possibility is that differences in solubility due to differences in H_2O content was responsible for these differences in activity. However the possibility cannot be ruled out that there was some physical degradation of the myosin caused by the freezing process. Such mechanical or sheer-type degradation has been observed with very large molecules such as DNA.

In aqueous solution at high ionic strength there have been no reports of any other type of ordered structure in myosin other than the α helix. The ir spectrum of myosin in D_2O at high salt concentration (0.3 M KCl) also indicates that the α-helical conformation is the only ordered conformation of myosin in solution at neutral pH. The infrared spectra of precipitated myosin also clearly indicate that under normal physiological conditions the α-helical conformation is the major ordered structure in myosin. However, with both specific solvent precipitation of the protein and with precipitation of the myosin at low pH, the presence of some β structure is indicated. Since the ir bands are broad, it is not possible to determine whether the β structure which is apparent under these conditions was originally part of the α-helical or the random conformation at pH 7. Similar studies of the possible formation of β structure in myosin in the solution phase are not possible since the myosin precipitates under the conditions in which some β structure is observed on the film.

It is possible that some β structure may be formed under acid conditions at higher pH. However, the principle feature characteristic of the β conformation only appears as a partially resolved shoulder at 1630 cm^{-1} on the dominant broad bands arising from α-helical and random conformations, and a small contribution from β structure would thus not be detectable.

Ir spectral changes have been used in a number of cases to investigate denaturation of proteins. Changes in secondary structure depend on the method of denaturation. Alkaline denaturation of β-lactoglobulin leads to a disordered state while in methanol denaturation a $\beta \rightarrow \alpha$ transition is observed (Timasheff and Gorbonoff, 1967). Hence the difference in observed conformations with various solvent precipitations of myosin is not surprising.

Any discussion of an *in vivo* role of β structure in the mechanism of muscular contraction is of course purely speculative. However, it is interesting to note that β structure could play a role in cross-bridge movement. In this regard phase changes (Flory, 1956) and conformational changes (Harrington, 1972) have already been postulated as involved in possible mechanisms for such movement. There is no experimental verification of these postulations. β structure has not previously been considered a possible alternative since none of the physical measurements of myosin in solution had indicated the presence of any β structure.

Huxley's (1969) model of muscular contraction postulated that the junction of light and heavy meromyosin acts as a flexible hinge to allow cross-bridge movement. Harrington (1972) postulated a helix–coil transition in the hinge region to allow simultaneous rotation and contraction of the lever arm. It is known that under physiological conditions the hydrolysis of ATP by myosin results in a burst of hydrogen ions (Finlayson and Tayler, 1969). It has been speculated that this burst of H^+ might change electrostatic balance thereby allowing a phase transition, or the rapid evolution of local concentration of ions might produce a helix–coil transition in the flexible hinge region (Harrington, 1972). Since a high concentration of H^+ ions during precipitation has been shown by the infrared spectra to result in the presence of some β structure in the myosin molecule, the rotation or change in the hinge region may be due to an $\alpha \rightarrow \beta$ (or random $\rightarrow \beta$) conversion. Conversion of part of the α helix to a parallel β conformation would result in an extension of length. Hence, a change in direction of orientation of the globular head of the molecular would occur. A change in orientation of the globular head of the molecule and an extension of length could also be caused by a random $\rightarrow \beta$ conversion. There are a number of cases known where stretching produced the β structure. Natural horse hair has the amide I and II bands characteristics of the α-helical and random conformations (Miyazawa and Blout, 1961), while in steam-stretched horse hair the antiparallel chain extended form is observed (Elliot *et al.*, 1962; Elliot and Bradbury, 1962). Antiparallel β structure has also been found in stream-stretched porcupine quill (Fraser and Suzuki, 1970). In synthetic polypeptides the conversion from $\alpha \rightarrow \beta$ structures is readily reversible in a number of cases (Davidson and Fasman, 1967) and such reversibility could be a part of the mechanism for muscular contraction and relaxation.

Acknowledgment

The authors thank Dr. G. Lee for helpful discussion and interpretation of the infrared spectra and Miss Marelle Law for the ir spectral determinations.

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Properties of Crystalline Kynureninase from *Pseudomonas marginalis*†

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ABSTRACT: The distribution of microbial kynureninase (L-kynurenine hydrolase, EC 3.7.1.3) among various strains was investigated and *Pseudomonas marginalis* was found to have the highest activity of enzyme, which was induced by the addition of L-tryptophan to the medium. The kynureninase was purified and crystallized from *Ps. marginalis*. The purified enzyme is homogeneous by the criteria of ultracentrifugation ($s^0_{20,w}$ = 5.87 S) and disc gel electrophoresis. The mol wt is 100,000, assuming a partial specific volume of 0.74. The enzyme exhibits absorption maxima at 280, 337, and 430 mμ. No appreciable spectral change was observed on varying the pH between 5.4 and 9.0. The holoenzyme can be resolved to the apoenzyme by incubation with hydroxylamine, L-alanine, and L-ornithine, and reconstituted by the addition of pyridoxal 5′-phosphate. One mole of pyridoxal 5′-phosphate is bound per mole of enzyme. The formyl group of pyridoxal 5′-phosphate is bound in an aldimine link to the ε-amino group of a lysine residue of the protein. The enzyme exhibits maximum reactivity at about pH 8.0; it is stable over the pH range 5.8–8.0. Neither D-kynurenine nor *N*-formyl-L-kynurenine is hydrolyzed by the enzyme. The Michaelis constants were determined as follows: L-kynurenine, 3.5×10^{-5} M, and pyridoxal 5′-phosphate, 2.3×10^{-7} M (0.065 M Tris-HCl buffer, pH 8.0). The enzyme activity was inhibited by carbonyl reagents and thiol reagents.

Kynureninase (L-kynurenine hydrolase, EC 3.7.1.3) is the enzyme which catalyzes the hydrolysis of L-kynurenine to L-alanine and anthranilate. Since the first observation of kynureninase in a mammalian liver extract (Kotake and Nakayama, 1941), several reports on the enzyme have appeared. The enzyme was partially purified from a pseudomonad (Hayaishi and Stanier, 1951), *Neurospora crassa* (Jakoby and Bonner, 1953a,b), and rat liver (Knox, 1953), and characterized. It has been also demonstrated that pyridoxal-5′-P[1] is required as a coenzyme for the reaction (Braunstein *et al.*, 1949; Wiss, 1949). Turner and Drucker (1971) recently presented evidence that two forms of tryptophan-inducible kynureninase exist in *Neurospora crassa*, which differ in kinetic properties, and in the reaction to pyridoxal-5′-P. Gaertner *et al.* (1971) also reported the presence of two types of kynureninase, *i.e.*, kynureninase and hydroxykynureninase; hydroxykynureninase is not inducible by tryptophan, but the other is, and their kinetic properties differ strikingly in their response to L-kynurenine and L-3-hydroxykynurenine.

In an attempt to elucidate the properties of kynureninase, a unique pyridoxal-5′-P enzyme catalyzing the hydrolytic β,γ cleavage of aryl-substituted γ-keto-α-amino acids, we have purified the enzyme to homogeneity from *Pseudomonas marginalis*, and crystallized the enzyme by the addition of ammonium sulfate (Moriguchi *et al.*, 1971a,b). In this paper more detailed studies on enzymological and physicochemical properties of crystalline kynureninase are described.

Experimental Section

Materials. L-Kynurenine sulfate was synthesized from L-tryptophan by the method of Warnell and Berg (1954). *N*-Formyl-L-kynurenine was prepared from L-kynurenine with

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[1] Abbreviations used are: pyridoxal-5′-P, pyridoxal 5′-phosphate; pyridoxamine-5′-P, pyridoxamine 5′-phosphate.