FURTHER EXPERIMENTS ON THE HEAT COAGULATION OF MYOSIN

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(Received June 12th, 1958)

SUMMARY

Earlier results on the heat coagulation of myosin have been extended. By a combination of successive heat coagulations and the effect of polyphosphates, preparations of very high adenylic deaminase activity have been obtained. The concentration of nucleic acid in the fractions surviving heat coagulation has been shown, particularly in those of highest enzymic activity. The relation of these fractions to the structure of myosin and the sub-units of other workers is discussed.

INTRODUCTION

In an earlier paper[1] the author examined the resistance of a portion of myosin to heat coagulation, as first reported by ENGELHARDT *et al.*[2]. The composition, end-groups and enzymic properties of the resultant proteins were examined. The heat-stable protein was resolved into several components. One of these, fraction P, had the solubility characteristics of myosin and an enhanced adenylic acid deaminase activity. Fraction D with three components, was soluble in solutions of low ionic strength, had low deaminase activity and had a high content of C-terminal groups. The present paper is an extension of this work. Polyphosphates have been found to influence the distribution of adenylic deaminase in the heat-resistant fragments. By successive heat coagulations it has been found possible to obtain very highly active preparations of adenylic deaminase containing ribonucleic acid.

EXPERIMENTAL

Myosin (three times precipitated) was prepared as described earlier[1].

Heat coagulations were carried out at 53° under conditions described earlier unless otherwise stated. The heat supernatants were resolved by dialysis against water into the insoluble and soluble fractions, P and D respectively.

Electrophoresis and enzymic assay (both by spectrophotometry and by Nesslerisation) were also carried out as before.

Chromatography of nucleotides was carried out in *n*-butyric acid–*N* NaOH–H_2O (69:21:10), ethanol–*M* ammonium acetate pH 3.8 (7.5:3) and *n*-propanol–conc. ammonia solution–water (60:15:25). Paper electrophoresis was carried cut in 0.05 *M* succinate buffer, pH 3.0.

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RESULTS

Ionic conditions for heat coagulation

In an earlier paper[1] heat coagulations were carried out in 0.5 *M* KCl, 2 *M* KCl giving a lower yield of fraction P. In the present work it was found that the series 0.5 *M*, *M*, 2 *M* KCl gave progressively increasing enzymic activity in the P fraction, but a maximum yield at *M* KCl. A standard medium of *M* KCl was therefore adopted, the pH being adjusted as before to 6.2.

Successive heat coagulations

If the P fraction which has already survived a heat coagulation, is redissolved and subjected to a second heat coagulation identical to that carried out on the original myosin, a second heat coagulum is formed. The proportion of protein remaining in solution (S_2) is higher than before and can again be resolved by dialysis against water into two parts. The insoluble and soluble fractions will be referred to as fractions P_2 and D_2. These show similar solubility properties to the P and D fractions. Electrophoresis of D_2 gave the same three-peak pattern as D.

The adenylic deaminase activities of the P_2 and D_2 fractions are higher than that of P from which they are derived. Unlike D, D_2 may be highly active (Table I).

TABLE I

YIELD, ACTIVITY AND OPTICAL PROPERTIES OF VARIOUS FRACTIONS

Experiment	*Fraction*	*No addition*			*Pyrophosphate*			*Tripolyphosphate*		
		Yield	Q_{NH_3}	*Absorption*	*Yield*	Q_{NH_3}	*Absorption*	*Yield*	Q_{NH_3}	*Absorption*
(1)	Myosin		40							
	P	7.9	119		10.9	132		12.1	96	
	D	4.8	5		5.5	72		6.1	118	
(2)	Myosin		27							
	P	9.7	104		9.7	152		9.1	109	
	D	4.5	7		4.5	54		5.4	74	
(3)	Myosin		60							
	P	15.5	121					18.3	39	
	D	5.7	30					5.0	120	
	P_2	42	89							
	D_2	11.8	393							
(4)	Myosin		138	0.80						
	P	12.0	483	2.1	10.5	255	2.0	15.0	208	1.6
	D	6.4	33	0.40	6.8	126	0.49	6.7	380	0.46
	P_2	33	880	3.7				38	530	3.1
	D_2	4.2	365	4.0				7.8	2000	5.7
(5)	P_2							33	415	3.0
	D_2							6.3	2050	4.7

Yield is expressed as % of protein in solution before heating.

Q_{NH_3} is expressed as μl NH_3/mg protein/h.

Absorption is O.D. max./mg protein. The absorption maxima were near 276 mμ for myosin and D and 259–260 mμ for P, P_2, D_2.

Experiments (1), (2) and first heating of (3) were in 0.5 *M* KCl.

Experiments (4), (5) and second heating of (3) were in *M* KCl.

Polyphosphates were 0.01 *M* in (1), (2), 0.05 *M* in (3) and 0.02 *M* in (4), (5).

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The effect of polyphosphates on the heat coagulation

The result of the heat coagulation was found to be affected by the presence of low concentrations (0.01–0.02 *M*) of pyrophosphate or tripolyphosphate. The presence of these substances usually increases the yield of P and D. The most marked effect however is on the deaminase activity of the fractions (Table I). There is an increase in the total activity accompanied by a displacement of activity from the P into the water-soluble D fraction. The latter effect is most marked with tripolyphosphate. The electrophoretic pattern of D is, however, unchanged. If a higher activity is desired in P, pyrophosphate may be used, while tripolyphosphate can be used to obtain high activity in the D fraction. This effect applies also to a second heat coagulation although as mentioned above, there is a tendency for high activity to appear in D_2 in the absence of polyphosphates.

Fractions of highest adenylic deaminase activity

The results of the two previous sections have been utilised in obtaining enzyme preparations of very high activity.

A first heat coagulation was carried out in the absence of polyphosphates, giving a concentration of activity in P. A second heat coagulation was then carried out on P in the presence of 0.02 *M* tripolyphosphate, giving a strong displacement of activity into D_2. By this method the best sample of D_2 obtained had an activity

$$Q_{NH_3}\,(23°) = 230{,}000\ (\mu l\ NH_3/mg\ protein/h)$$

measured by Nesslerisation at a substrate concentration of $4.3 \cdot 10^{-3}$ *M*. The corresponding P_2 fraction had a fifth of this activity.

Ultra-violet absorption spectra

While D has the normal u.v.-absorption of a protein, P has a maximum at approx. 260 mμ. This absorption is more pronounced in fractions of high enzymic activity. The spectra of P_2 and D_2 closely resemble those of nucleic acid or nucleotide. In nearly all cases the λ max is at 260 mμ. In Table I it can be seen that although there is no proportionality between absorption at 260 mμ and enzymic activity, there is a marked trend towards the association of high absorption and high activity.

Precipitation of P_2 and D_2 with trichloroacetic acid gave supernatants (ether extracted) of low absorption with no trace of a peak at 260 mμ. The precipitated D_2 could be redissolved in the original volume of water to give the same optical density at 260 mμ.

Absorbing material could be removed from the various fractions by passage through Deacidite-FF (OH form). The absorbing material could only be eluted at all rapidly with strong acids (4 *N* HCl or stronger) and then only incompletely.

If the proteins were precipitated with 10 % perchloric acid, little absorption was obtained immediately in the supernatant, but on standing in contact with the precipitate the absorption of the supernatant rose steeply. It appeared that the breakdown of a nucleic acid was involved. This was confirmed for myosin by subjecting a sample to cold 10 % perchloric acid first for 4½ hours then again for 6 days. The extracts were passed through Norit and the absorbing material eluted with ethanol–ammonia. Paper chromatography of both extracts, followed by spectral measurements and phosphate determinations revealed the presence of adenylic, guanylic, cytidylic and

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uridylic acid in each. The 4½ h extract contained in addition adenosine diphosphate, characterised by the above criteria and paper electrophoresis. The total nucleic acid and nucleotide content of myosin was approx. 0.4% of which less than 10% was nucleotide.

Relation of the heat-stable fractions to the meromyosins

A sample of myosin was subjected to tryptic fission under the conditions used by SZENT-GYÖRGYI[3]. L-meromyosin was precipitated by dialysis (without storage) and washed. The H-meromyosin was used without purification for enzymic experiments but was fractionated with ammonium sulphate for spectral measurements.

The relative deaminase activities (myosin = 1) of the L- and H-meromyosins were 1.3 and 0.6 respectively. Correcting for a 15% contamination of H- with L-meromyosin[3] the value for H would be reduced to 0.5.

Unpurified H-meromyosin gave low and ill-defined maxima near 260 mμ in short (2 min) and long (3 day) term 10% perchloric acid extracts, while ammonium sulphate fractionated material gave no maxima. A short term perchloric acid extract of L-meromyosin gave a well defined maximum at 259 mμ and a subsequent long term extract at 260 mμ, three times greater than the first. It appears that the nucleotide and nucleic acid content of the myosin passes entirely into the L-meromyosin.

DISCUSSION

The effect of a first heat coagulation is to dissociate from myosin a protein P having the solubility characteristics of myosin with enhanced nucleic acid and deaminase content, together with three fragments D soluble at low salt concentrations. A heat coagulation of P gives rise to P_2, D_2 fractions similar in properties to their earlier counterparts but in greater yield and having still higher nucleic acid and enzymic content. This is particularly true for D_2 when tripolyphosphate is present. The D components appear, from their low sedimentation and high diffusion rates and the high end-group content of the major component, to be of low molecular weight[1]. They may therefore represent fundamental sub-units of myosin, progressively liberated during the successive heat treatments by loosening of the molecular structure.

While this work was being carried out, MIHALYI, LAKI AND KNOLLER[4] reported the presence of ribonucleic acid (RNA) in myosin in amounts comparable to that found in the present work. The adenine nucleotide they found present in small quantity would correspond to the ADP found here in short-term perchloric acid extracts of myosin. The fact that RNA is always concentrated in the fractions surviving the heat treatment suggests that it may exert a stabilising effect on the portion of the protein to which it is attached. ZUBAY AND DOTY[5] report the stabilisation of serum albumin to heat coagulation by small amounts of yeast deoxyribonucleic acid, although their ionic conditions were rather different.

Two reports have recently been made of the preparation of highly active adenylic deaminase. NIKIFORUK AND COLOWICK[6] purified SCHMIDT'S enzyme by alumina, ammonium sulphate and paper fractionations. The activity of their best preparation was equivalent to a Q_{NH_3} of 30,000 (25°) at a substrate concentration of $4 \cdot 10^{-3}$ *M*. LEE[7] used a heat coagulation of actomyosin followed by ethanol, ammonium sulphate and calcium phosphate gel fractionations to obtain a crystalline enzyme with all the

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properties of a pure protein. His best preparation had an activity of 2,500,000 (30°) at substrate-saturation or 1,900,000 at $4.3 \cdot 10^{-3}$ *M* substrate concentration. The value of 230,000 (23°) obtained in this work at that concentration is thus well below that of the crystalline enzyme but higher than that of NIKIFORUK AND COLOWICK. It does, however, offer a simple and rapid method for the preparation of enzyme of high activity from myosin.

The activity of the best fractions as reported earlier is about a hundred times greater when determined by Nesslerisation at $4.3 \cdot 10^{-3}$ *M* substrate concentration than when determined spectrophotometrically at $5.2 \cdot 10^{-5}$ *M*. This indicates that the Michaelis-Menten constant (K_M) of $1.4 \cdot 10^{-3}$ *M* found by LEE is more correct for these fractions than the values of $6 \cdot 10^{-5}$ (ref. [6]) and $5 \cdot 10^{-4}$ (ref. [7]) reported for other preparations.

The crystalline enzyme of LEE shows an absorption maximum at approx. 276 mμ although material with a maximum at 265 mμ could be extracted with perchloric acid. It appears that the nucleic acid of the present preparations is not an essential part of the enzyme. The lack of a precise relation between absorption and activity also favours this view. The nucleic acid may have a peculiar affinity for the enzyme protein, thus exerting a protective effect.

The deaminase activity of myosin has already been found by NEKHOROCHEFF, DONDON AND GOUSSEF[9] to be split between the meromyosins with the greatest concentration in the L-meromyosin. The activities of L and H relative to myosin were found by these authors to be 1.3 and 0.5 respectively which agrees very closely with the results obtained here. The presence of appreciable activity in both fractions indicates that the enzyme must be bound to several sites on the myosin. The nucleic acid and nucleotide absorption on the other hand passes almost entirely into the L-meromyosin. Although the mild heat treatment used in this work would not be expected to split peptide bonds as trypsin probably does in the preparation of meromyosins, it appears that the P and P_2 fractions have in common with L-meromyosin a salt solubility similar to myosin, enhanced deaminase activity and nucleic acid content, and a resistance to heat coagulation. Undoubtedly the L-fraction of SNELLMAN[10] obtained from ammonium sulphate fractionation of myosin at 20° is also related.

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Erratum

In the earlier paper in this journal[1] the labelling of the ultracentrifuge diagrams in Fig. 2, p. 516 was reversed, *i.e.* the upper diagram should refer to fraction D and the lower to fraction P.