BBA 45931

REACTION OF MYOSIN WITH SALICYLALDEHYDE

I. THE EFFECT OF SALICYLALDEHYDE ON THE PHYSICOCHEMICAL PROPERTIES OF MYOSIN

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SUMMARY

The specificity and nature of the reaction between salicylaldehyde and myosin and the effect of salicylalation on the molecular parameters of myosin were studied. The following observations were made.

1. The reaction of salicylaldehyde with the lysyl residues of myosin is specific, since no salicylaldehyde is bound if the lysyl residues of myosin are trinitrophenylated.

2. Salicylaldehyde is bound by myosin through the formation of an azomethine linkage (Schiff's base). This was established from the measured difference absorption spectrum of the myosin–salicylaldehyde complex.

3. Three groups of lysyl residues can be distinguished with respect to the reaction with salicylaldehyde, namely, (a) residues with high association constant ($K_{ass} = 1.8 \pm 0.9 \cdot 10^5\ M^{-1}$), (b) residues with moderate association constant ($K_{ass} = 2.2 \cdot 10^3\ M^{-1}$) and (c) residues that react with salicylaldehyde only after the denaturation of the protein. Their numbers could be estimated as 10 ± 5, 130 ± 5 and 260 ± 5 per mole myosin, respectively. The first group of residues was found to be absent from heavy and light meromyosin, the proteolytic fragments of myosin.

4. The reaction is reversible. The complex formation rate constant, evaluated from the formula for second order reaction, is 2.2 $sec^{-1} \cdot M^{-1}$, and the decomposition rate constant for first order reaction is $1.1 \cdot 10^{-3}$ sec^{-1} at 22°.

5. The reaction is pH dependent, the reaction yield increasing at higher pH.

6. The solubility of myosin at low ionic strength decreases with increasing degree of salicylalation at slightly alkaline pH.

7. The intrinsic viscosity of myosin does not change on salicylalation.

8. A second peak due to polymerization appears on the sedimentation profile of the protein if more than 70 lysyl residues are salicylalated per mole of myosin.

INTRODUCTION

The results of a thorough experimental study of the effect of 2,4,6-trinitrobenzenesulphonate (TBS) on the lysyl residues of myosin suggested that some of the

Abbreviation: TBS, 2,4,6-trinitrobenzenesulphonate.

lysyl residues have a critical importance in the structure of the enzymic centre of myosin ATPase (ATP phosphohydrolase, EC 3.6.1.3)[1]. It seemed of interest therefore to investigate the effect of other amino group reagents on the molecular and enzymic properties of myosin.

Salicylaldehyde was chosen for the experiments, since it has already been used for the modification of the lysyl ε-amino groups of cytochrome *c* by WILLIAMS AND JACOBS[2,3] and for some other proteins by MEANS AND FEENEY[4]. No details showing the specificity of the reaction or concerning the nature of the protein–salicylaldehyde bond have been reported by the authors. These two aspects of the reaction were therefore extensively investigated along with its effects on the molecular properties of myosin. The results of these investigations are reported in the present paper but the experimental data on the changes caused by the reagent in the biological (enzymic and actin binding) properties of myosin will be discussed in a subsequent report[5].

MATERIALS AND METHODS

Myosin was prepared essentially as described by PORTZEHL *et al.*[6] and dissolved after the last precipitation in 0.5 M KCl–0.02 M borate buffer (pH 7.4) then centrifuged at 105000 × *g* in a Spinco L50 preparative ultracentrifuge for 1 h. Fresh myosin, not older than 5 days, was used throughout. In the calculations the molecular weight of myosin was taken to be $5 \cdot 10^5$ g (ref. 7). Heavy and light meromyosin were prepared essentially as described by SZENT-GYÖRGYI[8] but after digestion the proteolysis was inhibited by the addition of diisopropylfluorophosphate up to 0.01 M final concentration[9]. Salicylaldehyde was obtained from Merck and polylysine (mol. wt. 195000) from Sigma. Other reagents were obtained from Reanal. Reagent grade products were used throughout.

The buffer solution used was, if not stated otherwise, 0.5 M KCl–20 mM borate buffer referred to as KCl–borate solution.

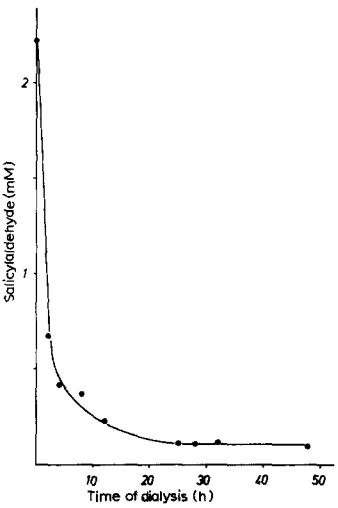
Protein content was measured by the biuret method of GORNALL *et al.*[10] and by the microbiuret method[11].

Salicylaldehyde treatment, if not stated otherwise, was performed by using a stock solution of 30 mM salicylaldehyde containing 5 % ethanol from which different quantities were added to a KCl–borate solution (pH 8) containing 10 mg of protein per ml. The test solution was incubated at 0° for 1 h, then dialysed against 100 vol. of the KCl–borate solution at 3° for 24 h without changing the dialysing solution. This time was needed to reach equilibrium between the free salicylaldehyde inside and outside the dialysis bag. The time curve of a characteristic equilibrium dialysis is shown in Fig. 1. The concentration of salicylaldehyde was estimated by the method of WILLIAMS AND JACOBS[2], and the bound salicylaldehyde concentration was evaluated by subtracting the value measured outside the dialysis bag from that estimated inside the bag. The enhancement of the normally slow decomposition of salicylaldehyde in aqueous solution by the presence of proteins (Fig. 2) was taken into account in the evaluation of the measurements.

The difference spectra were measured by an Opton PMQ II spectrophotometer and the other spectra by a Spectromom 360 spectrophotometer. For the optical rotatory dispersion measurements an Opton REP spectropolarimeter was used. The intrinsic viscosity was measured by an Ubbelohde viscometer. The shearing-stress

dependence of the reduced viscosity was determined on an Ostwald viscometer using different volumes of the test solution to vary the shearing stress which was evaluated by the method of YANG[12] from the difference between the solution levels in the two capillaries. All viscosity measurements were carried out at 20°.

The sedimentation experiments were performed in a Beckman Model EHT analytical centrifuge.



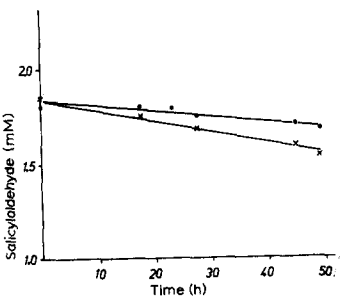


Fig. 1. Equilibrium dialysis of myosin–salicylaldehyde complex. 5 mg of myosin per ml and 2.2 mM salicylaldehyde were incubated in KCl–borate solution (pH 7.6) at 0° for 1 h, then dialysed against 100 vol. of the KCl–borate solution (pH 7.6) at 3°. Ordinate: salicylaldehyde concentration (mM) in the dialysis bag.

Fig. 2. Decomposition of salicylaldehyde in aqueous solution with and without the addition of myosin. 1.85 mM salicylaldehyde was incubated in KCl–borate solution (pH 8.0) at 3°. ●, without myosin; ×, with 10 mg of myosin per ml.

RESULTS

Specificity and nature of the salicylaldehyde–myosin reaction

To prove the specificity of the reaction between salicylaldehyde and the amino groups of myosin it was shown that salicylaldehyde does not react with other reactive groups of myosin. The amino groups of myosin were therefore trinitrophenylated with TBS which, as shown by OKUYAMA AND SATAKE[13] specifically reacts with the amino groups of proteins. The salicylaldehyde binding was then studied by means of an equilibrium dialysis experiment with trinitrophenylated myosin, native myosin and myosin treated as for trinitrophenylation but without the addition of TBS. The results listed in Table I show that salicylaldehyde does not react with trinitrophenylated myosin. That more salicylaldehyde was bound by Sample 3 than by Sample 2 was probably due to the incubation in alkaline pH at 37° for 4 h leading to the partial denaturation of myosin and thus to the availability of new lysyl residues for the salicylaldehyde reaction.

On the reaction of salicylaldehyde with amines a difference in spectral absorbance arises owing to the formation of a Schiff's base, *i.e.* azo-methine linkage[14]. The difference spectra observed as a result of the formation of myosin–salicylaldehyde

TABLE I

REACTION OF SALICYLALDEHYDE WITH NATIVE AND TOTALLY TRINITROPHENYLATED MYOSINS

5 mg of myosin per ml were incubated in the presence of 0.25% TBS and 3% $NaHCO_3$ at 37° for 4 h. Excess TBS was removed by dialysis against KCl–borate solution (pH 8) at room temperature for 48 h, the dialysing solution being changed several times. After the removal of TBS the dialysis was continued against 1 vol. of a solution of 2.5 mM salicylaldehyde in KCl–borate solution (pH 8) at 3° for 40 h until equilibrium was reached between the solutions outside and inside the dialysis bag. Samples 1 and 2 were treated only with salicylaldehyde under the same conditions. Bound salicylaldehyde was evaluated as described in MATERIALS AND METHODS, except for Sample 4 where the salicylaldehyde concentration was not measured inside the dialysis bag because of interference from the trinitrophenyl groups. In this sample bound salicylaldehyde was obtained by doubling the difference between the salicylaldehyde outside the dialysis bag of Sample 1 and that of Sample 4.

Contents of the dialysis bag	*Salicylaldehyde concn. (mM)*		*Moles of bound salicylaldehyde per mole myosin*
	Inside the dialysis bag	*Outside the dialysis bag*	
(1) Buffer, without protein	1.22	1.24	0.0
(2) Native myosin	1.50	0.91	59
(3) Myosin treated as 4, in absence of TBS	1.70	0.76	94
(4) Trinitrophenylated myosin	Not measured	1.25	0.0

and polylysine–salicylaldehyde complexes are shown in Fig. 3. The maxima were at 285 and 440 mμ for the polylysine–salicylaldehyde and at 280 and 415 mμ for the myosin–salicylaldehyde complex. These values compare well with 260 and 410 mμ measured by FERGUSON AND KELLY[14] after the reaction of salicylaldehyde with ethylenediamine.

The reaction of salicylaldehyde with myosin was found to be reversible by the dialysis of the complex against KCl–borate solution (pH 7.6). A gradual decrease in bound salicylaldehyde was observed in the aliquots taken each time the dialysing solution was changed. The decomposition of the complex with few salicylaldehyde

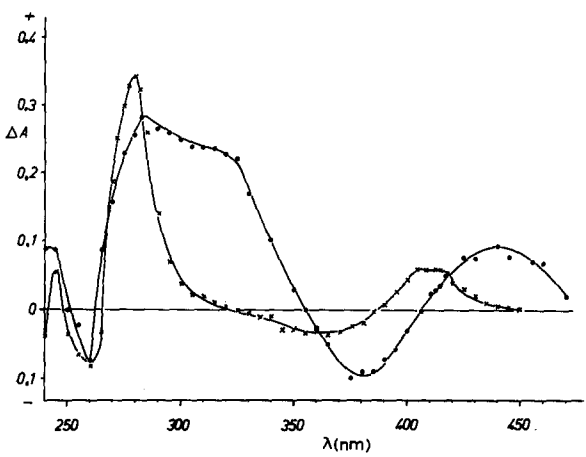


Fig. 3. Difference spectra of the salicylaldehyde–polylysine and salicylaldehyde–myosin complexes. ●, 0.3 mM salicylaldehyde + 0.022 mg of polylysine per ml; ×, 0.2 mM salicylaldehyde + 1 mg of myosin per ml. Both were incubated at room temperature in a solution of 0.5 KCl–40 mM borate buffer (pH 8) for 1 h before the difference spectra were measured.

was very slow. 1 mole of salicylaldehyde per mole of myosin was found to be bound even after dialysis for 250 h (Fig. 4).

The number of lysyl residues available for reaction with salicylaldehyde and the association constant K_{ass} of the myosin–salicylaldehyde complex were estimated from the data of the equilibrium dialysis and the difference in absorbance using the method of Scatchard[15]. The concentration of bound salicylaldehyde as a function of total salicylaldehyde concentration was evaluated from the equilibrium dialysis data (Fig. 5). The difference in absorbance at 280 mμ was then measured (Fig. 6) on the complexes treated with the same salicylaldehyde concentrations under the same conditions as used for the equilibrium dialysis. The molar absorption coefficient was evaluated from the values of bound salicylaldehyde and difference in absorbance at 280 mμ and found to be $\Delta\varepsilon = 4900$. This value was used for the calculation of the

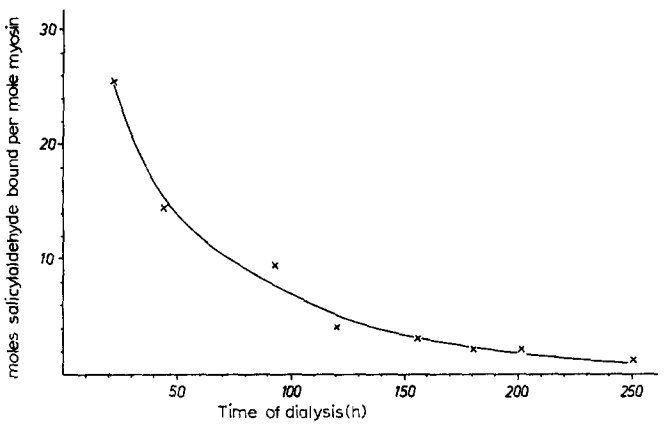


Fig. 4. Removal of salicylaldehyde from the salicylaldehyde–myosin complex by dialysis. 3 mM salicylaldehyde + 5 mg of myosin per ml were incubated in KCl–borate solution (pH 7.6) for 1 h at 0°. The solution was then dialysed against 100 vol. of KCl–borate solution (pH 7.6). The dialysing solution was changed each time an aliquot was taken.

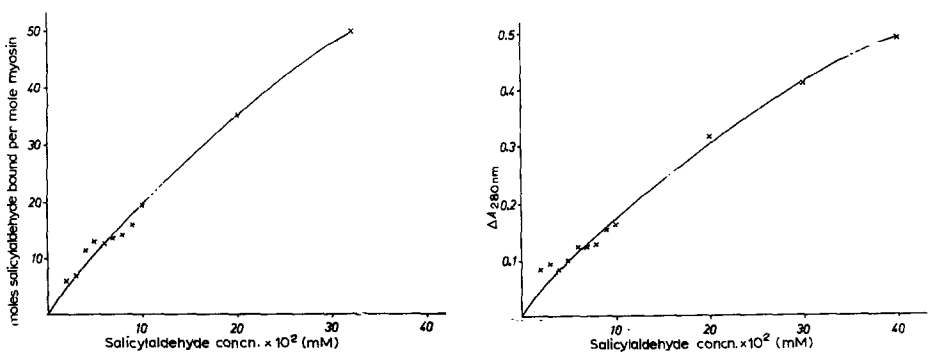


Fig. 5. Dependence of the salicylalation of myosin on salicylaldehyde concentration. 1 mg of myosin per ml was dialysed against 100 vol. of KCl–borate solution (pH 8) to which salicylaldehyde was added in different concentrations and incubated at 22° for 24 h.

Fig. 6. Dependence of the difference in absorbance on salicylaldehyde concentration. 1 mg of myosin per ml was incubated with salicylaldehyde at different concentrations in KCl–borate solution (pH 8) at 22° for 1.5 h and the difference in absorbance was measured at 280 mμ.

bound salicylaldehyde concentration in the experiments in which only the absorption difference was measured. The Scatchard plot made by using the experimental data shown in Figs. 5 and 6 is shown in Fig. 7. It is apparent from Fig. 7 that there are at least two types of binding site in myosin capable of binding salicylaldehyde. Both sites are thought to be lysyl residues which react with salicylaldehyde through the formation of a Schiff's base. This can be inferred because essentially the same plot was obtained from the direct measurement in equilibrium dialysis as from the difference in spectral absorbance due to formation of Schiff's base. The K_{ass} and the number of binding sites (n) could be evaluated from the Scatchard plot for the "weak type"

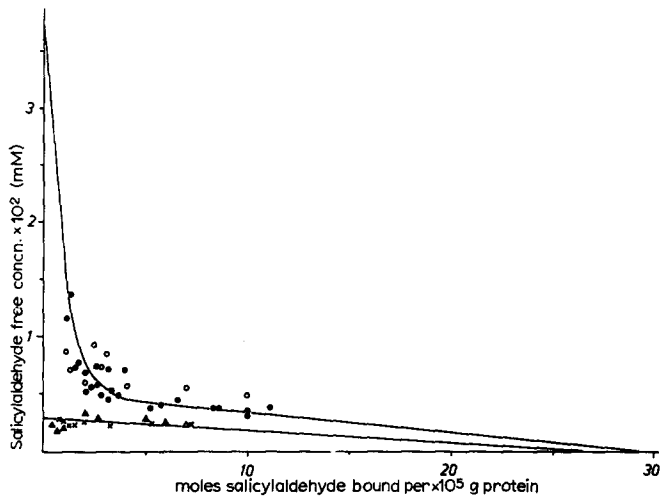


Fig. 7. Binding of salicylaldehyde by myosin, heavy and light meromyosin (Scatchard plot). ○, for myosin (bound salicylaldehyde measured directly, Fig. 5); ●, for myosin (salicylaldehyde bound evaluated from the $A_{280\ m\mu}$, Fig. 6); ×, for heavy meromyosin (evaluated from Fig. 11); △, for light meromyosin (evaluated from Fig. 11).

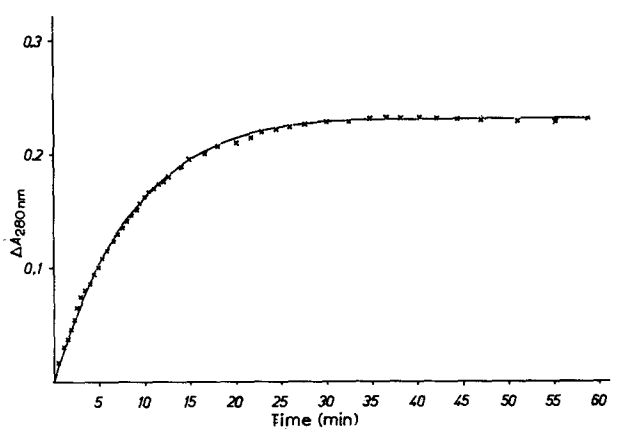


Fig. 8. Rate of the salicylaldehyde–myosin complex formation. The increase in the difference of absorbance at 280 mμ was measured at 22° upon addition of myosin in 1.05 mg/ml final concentration to a solution containing 0.15 mM salicylaldehyde in KCl–borate (pH 8). The continuous line is a theoretical curve plotted with the use of k_1 and k_{-1} evaluated from the initial velocities. Crosses, measured values.

as $K_{ass} = 2.2 \cdot 10^3\ M^{-1}$ and $n = 130 \pm 5$ per mole of myosin. Owing to the uncertainty of the measurement at low salicylaldehyde concentration, only a rough estimation was possible for the "strong" type, as $K_{ass} = 1.8 \cdot 10^5 \pm 0.9 \cdot 10^5\ M^{-1}$ and $n = 10 \pm 5$ per mole of myosin.

The myosin–salicylaldehyde complex formation was followed up from the increase in the absorption difference at 280 mμ measured after the addition of salicylaldehyde (Fig. 8). The rate constant of the myosin–salicylaldehyde complex formation, k_1, was evaluated from the measured initial velocity of the reaction using the formula for second order reaction of the form

$$k_1 = \frac{1}{t(a-b)} \ln \frac{b(a-x)}{a(b-x)}$$

where t is the time in sec, a and b are the molar concentrations of the available lysyl residues and of salicylaldehyde, respectively, at the beginning of the reaction and x is the molar concentration of the complex formed in time t. The calculation gives $k_1 = 2.2\ sec^{-1} \cdot M^{-1}$.

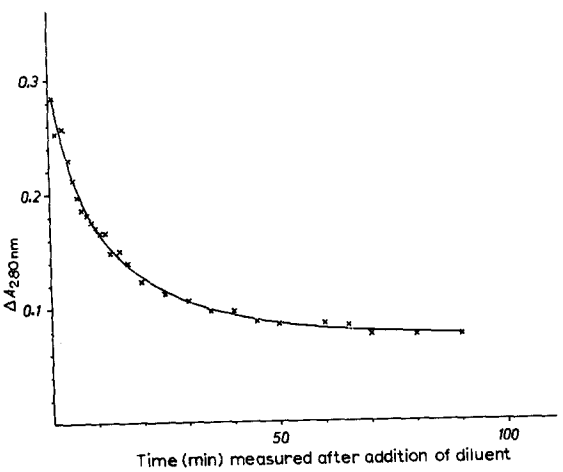


Fig. 9. Rate of the salicylaldehyde–myosin complex decomposition. The solution of 2 mg of myosin per ml, incubated with 1.3 mM salicylaldehyde–borate solution (pH 8) at 22° for 1.5 h, was diluted by 10 vol. of KCl–borate (pH 8) and the decrease in $\Delta A_{280\ m\mu}$ was measured.

The decomposition of the complex was evaluated from the observed decrease in the absorption difference at 280 mμ after dilution (Fig. 9) which shifts the equilibrium in the direction of dissociation. The rate constant of decomposition, k^{-1}, was calculated from the measured initial velocity using the formula for first order reaction

$$k_{-1} = \frac{1}{t} \ln \frac{a}{a-x}$$

where a stands for the molar concentration of the myosin–salicylaldehyde complex at the beginning of the reaction and x for that of the complex decomposed in time t. The calculation gives $k_{-1} = 1.1 \cdot 10^{-3}\ sec^{-1}$.

The curve plotted in terms of the above two rate constants reproduces essentially the experimental points of Fig. 8.

The association constant evaluated from the ratio k_1/k_{-1} as $K_{ass} = 2 \cdot 10^3$ M^{-1} is in good agreement with the value calculated from the Scatchard plot for the "weak" binding site.

The reaction of myosin with salicylaldehyde was found to be markedly pH dependent (Fig. 10). The salicylalation sharply increased with increasing pH. At high pH the viscosity of the myosin solution increased with the bound salicylaldehyde concentration. At pH 9 for 200 moles of salicylaldehyde per mole of myosin even gelification was observed.

The complex formation of salicylaldehyde with the proteolytic fragments, heavy and light meromyosin, of myosin was also measured. The dependence of the complex formation on salicylaldehyde concentration is shown in Fig. 11. The salicylaldehyde binding of the proteolytic fragments was found to be lower than that of the parent molecule if calculated with the binding to 10^5 g of each protein at the relatively low salicylaldehyde concentrations used. The Scatchard plots of heavy and light meromyosin obtained from the data of Fig. 11 are shown in Fig. 7. For the proteolytic fragments no distinction could be made between the binding sites. Calculating with molecular weights of $3.6 \cdot 10^5$ g for heavy meromyosin and $1.47 \cdot 10^5$ g for light mero-

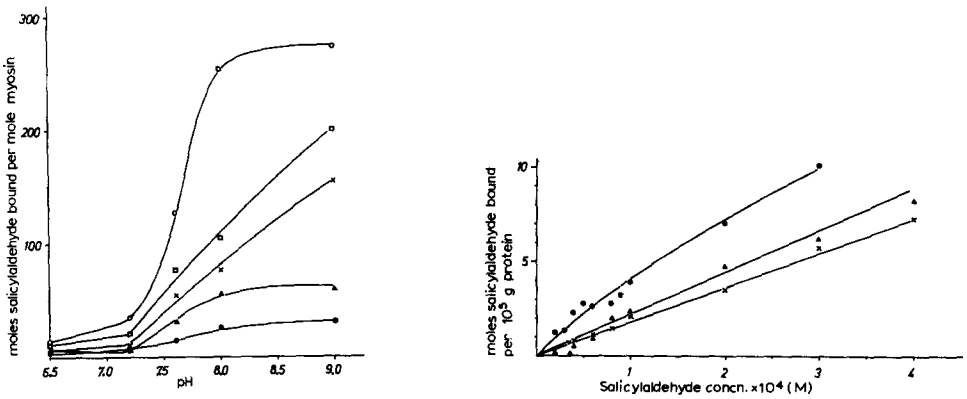


Fig. 10. Effect of pH on the formation of salicylaldehyde–myosin complex. 10 mg of myosin per ml were incubated at 3° for 1 h with salicylaldehyde at different concentrations in 0.5 M KCl–0.016 M borate buffer at pH 7.6, 8.0 or 9.0 and in 0.5 M KCl–0.016 M Tris–maleate buffer at pH 6.5 or 7.2. Salicylaldehyde concentrations (mM): ○, 10; □, 7; ×, 5; △, 3; ●, 1. The solution was then dialysed against 100 vol. of the buffer used for incubation + 0.5 M KCl at 3° for 24 h.

Fig. 11. Dependence of the salicylalation of myosin, light and heavy meromyosin on salicylaldehyde concentration. ●, myosin; △, light meromyosin; ×, heavy meromyosin; in concentrations of 1 mg/ml were incubated with salicylaldehyde at different concentrations in a KCl–borate solution (pH 8) at 22° for 1.5 h and the $\Delta A_{280\,m\mu}$ was measured.

myosin, the Scatchard plots yield $K_{ass} = 980$ M^{-1} and $n = 91.8$ per mole for heavy meromyosin, and $K_{ass} = 1120$ M^{-1} and $n = 42.1$ per mole for light meromyosin. The sum of the binding sites estimated for the two proteolytic fragments is roughly equal to the estimated number of the weak binding sites of myosin, but the values of K_{ass} are lower than estimated for the weak binding sites of the parent molecule, while "strong" sites do not seem to exist.

Effect of salicylalation on the physicochemical properties of myosin

The low solubility of myosin at low ionic strength was expected to be further reduced by the introduction of hydrophobic groups, as observed for the cytochrome *c*–salicylaldehyde reaction[2,3]. However, a decrease in solubility was observed on the reaction with salicylaldehyde only at pH 8–9 (Table II). It seems that at slightly acidic and neutral pH's the effect of the introduction of hydrophobic groups is balanced by the decrease in positive charges due to salicylalation of the lysyl residues

TABLE II

EFFECT OF SALICYLALATION ON THE SOLUBILITY OF MYOSIN AT LOW IONIC STRENGTH

10 mg of myosin per ml in 0.5 M KCl were diluted by 12 vol. of 15 mM acetate buffer at pH 6 or borate buffer at pH 7, 8 or 9. After standing for 10 min the solutions were centrifuged at 21 500 × *g* for 20 min. All procedures were carried out at 0°. The protein concentration of the supernatant was measured by the method of Folin[18]. The solubility was evaluated from the ratio of the protein content of the supernatant to the total protein content.

Moles of bound salicylaldehyde per mole myosin	*Solubility of myosin at pH*			
	6	*7*	*8*	*9*
0.0	0.0067	0.0142	0.156	0.690
9.1	0.0070	0.0164	0.140	0.526
21.1	0.0070	0.0140	0.124	0.440
39.9	0.0072	0.0152	0.063	0.138
52.6	0.0066	0.0139	0.039	0.067

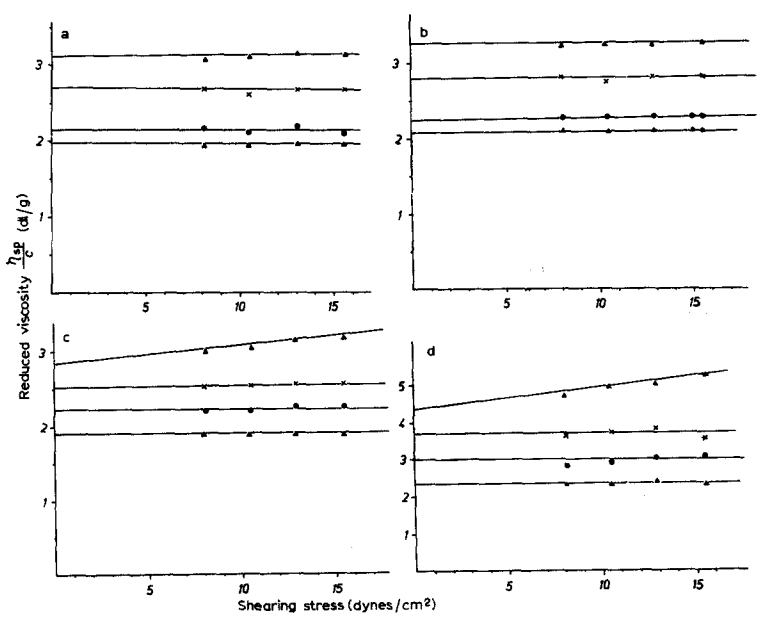


Fig. 12. Dependence of the reduced viscosity of native and salicylalated myosin on the shearing stress. (a) Native myosin; (b) 4.57; (c) 8.5; (d) 97.0 moles of bound salicylaldehyde per mole myosin. Myosin concentration (mg/ml): △, 1.13; ●, 2.11; ×, 2.98; ▲, 5.05.

which increases the net value of the negative charges of the myosin molecule. At higher pH the amino groups are partly deprotonated and less charge is lost by the molecule on salicylalation, thus the effect of the introduction of hydrophobic residues can assert itself.

The optical rotatory dispersion measurements (Table III) showed that the α-helix content of myosin did not change on salicylalation up to 60 moles of salicylaldehyde per mole of myosin. Above this value an approx. 10 % decrease in helicity was always observed.

The effect of salicylaldehyde reaction on the shape of the myosin molecule was studied by viscometry. The dependence of the reduced viscosity on the shearing stress was measured in order to establish whether or not the modified samples have Newtonian viscosity (Fig. 12). The reduced viscosity was found to depend on the shearing

TABLE III

EFFECT OF SALICYLALATION ON THE HELICAL CONTENT OF MYOSIN

Optical rotatory dispersion measurements were carried out in the range from 270 to 235 mμ. Helicity was calculated by the method of SIMMONS *et al.*[17] from the rotation at 233 mμ. Conditions of the determination: 0.5 mg of myosin per ml KCl–borate solution (pH 8).

Moles of bound salicylaldehyde per mole myosin	*Helicity* (%)
0.0	53.5
6.6	54.0
17.0	53.5
42.9	54.0
59.3	50.0
96.0	46.0

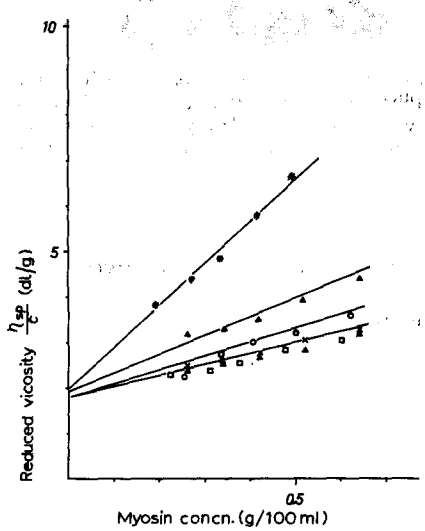


Fig. 13. Reduced viscosity *vs.* myosin concentration. △, native myosin; ×, 4.0; □, 11.0; ○, 36.0; ▲, 71.0; ✱, 105.0 moles of bound salicylaldehyde per mole myosin.

stress (*i.e.* it was non-Newtonian) at the higher degree of salicylalation only in the solutions with a higher protein concentration than 5 mg/ml. The intrinsic viscosity measured in the solutions having Newtonian viscosity did not change essentially on salicylalation, but the slopes of the curves increased with increasing complex formation above 11 moles of bound salicylaldehyde per mole of myosin (Fig. 13).

The sedimentation patterns of native myosin and the myosin–salicylaldehyde complexes are shown in Fig. 14. Only a simple homogeneous peak is seen in the sedimentation patterns of native myosin and of the complexes with few moles of salicyl-

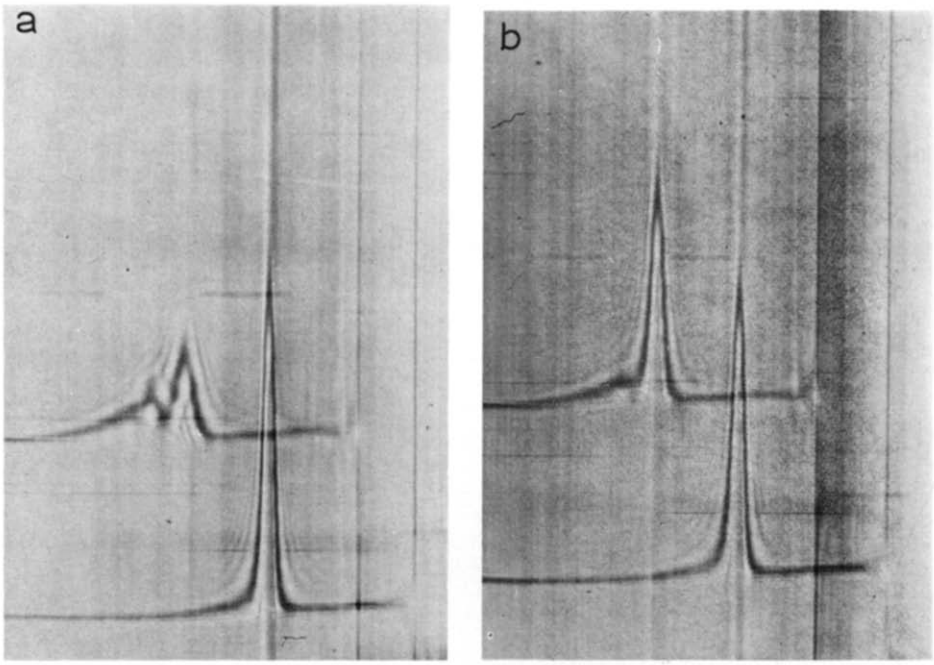


Fig. 14. Sedimentation profiles of native and salicylalated myosins. Protein concentration: 2.62 mg/ml. Centrifuged at 50740 rev./min at 20°. Photographed at 55 min after reaching full speed. Bar angle 70°. a. Upper curve, 105 moles of salicylaldehyde bound per mole myosin; lower curve, native myosin. b. Upper curve, 71.2; lower curve, 11.1 moles of salicylaldehyde bound per mole myosin.

TABLE IV

s_{20} VALUES OF THE COMPONENTS OBSERVED IN THE SEDIMENTATION PROFILES OF MYOSIN AND MYOSIN–SALICYLALDEHYDE COMPLEXES

s_{20} values were calculated from the sedimentation profiles in Fig. 14.

Moles of bound salicylaldehyde per mole myosin	s_{20}	
	First peak	*Second peak*
0.0	4.87	—
11.1	5.01	—
71.2	5.10	6.3
105.0	5.61	6.8

aldehyde per mole of myosin. A second peak appeared at higher degrees of salicylalation indicating the polymerization of myosin. The size of the second peak increased with the degree of salicylalation showing the dependence of polymerization on salicylalation. Polymerization was observed also by WILLIAMS AND JACOBS[3] on the salicylalation of cytochrome *c*. The values of s_{20} calculated from the sedimentation patterns (Table IV) increased with the measure of the salicylaldehyde reaction. The increase was greater than to be expected from the Johnston–Ogston effect. The increase in s_{20} with higher degrees of salicylalation can be attributed to the increasing contribution from molecular interactions.

DISCUSSION

Salicylaldehyde was found to react specifically with the lysyl residues of myosin through the formation of a Schiff's base. With respect to the reaction with salicylaldehyde the 400 lysyl residues of myosin can be divided into three groups. (1) Residues with high affinity for salicylaldehyde ($K_{ass} = 1.8 \cdot 10^5 \pm 0.9 \cdot 10^5\ M^{-1}$) which are relatively few ($n = 10 \pm 5$ moles per mole of myosin). (2) Residues with moderate affinity for salicylaldehyde ($K_{ass} = 2.2 \cdot 10^3\ M^{-1}$) and much more numerous ($n = 120 \pm 5$ moles per mole of myosin). The residues of Groups 1 and 2 are available for salicylaldehyde reaction without the denaturation of myosin. Since no change was observed in the value of k_1 during the progress of salicylaldehyde complex formation (Fig. 8), the reaction of the two types of residue must have essentially the same value of k_1. The value of k_{-1}, however, is much smaller for Group 1 considering that some of the bound salicylaldehyde could not be removed even after dialysis for 250 h (Fig. 3). Group 3 contains residues that react with salicylaldehyde only after the denaturation of myosin (see Table I). It is difficult to distinguish them from those of Group 2, seeing that the binding of salicylaldehyde by itself leads to the denaturation of myosin as can be inferred from the gelification and optical rotatory changes *etc.* at high degrees of salicylalation. The lysyl residues of cytochrome *c* could not be differentiated with respect to the reaction with salicylaldehyde since all the lysyl residues were available for salicylaldehyde reaction in the native state of this protein[2,3].

In the proteolytic fragments of myosin, heavy and light meromyosin only the lysyl residues of Groups 2 and 3 were identified. A possible explanation of the absence of the residues belonging to Group 1 from heavy and light meromyosin could be that these residues are located in that part of the myosin molecule which is split into peptides during the formation of the proteolytic fragments[18]. An alternative explanation could be that a conformational change takes place on the proteolysis of myosin leading to a decrease in the affinity for salicylaldehyde owing to an alteration in the environment of the binding sites. This latter assumption is supported by the observation that the residues of Group 2 had a lower value of K_{ass} in the proteolytic fragments than in the parent molecule.

The solubility is the first of the molecular parameters that was observed to change as the degree of salicylalation increased. It seems possible that the introduction of a hydrophobic group is not the only cause of the decrease in solubility observed for alkaline pH. The solubility may also be reduced by the polymerization of myosin at high degrees of salicylalation (Fig. 14). If more than 150 moles of salicylaldehyde

are bound per mole of myosin, the protein becomes insoluble even at high ionic strength.

A second peak due to polymerization was observed on the sedimentation profiles of the complexes with 71 or 101 moles of salicylaldehyde per mole of myosin. In spite of the polymerization, no appreciable change could be observed in the intrinsic viscosity of these samples (Fig. 13). This implies that unstable, concentration dependent polymers form on salicylalation. Further evidence of this is the increase in the slope of the reduced viscosity *versus* concentration curve with increasing salicylalation, and the high value of the Huggins constant (k') which according to YANG[12] shows that the solute–solvent interaction is poor, enabling the solute molecules to interact with one another forming unstable, concentration-dependent aggregates. The concentration-dependent interaction of the myosin molecules is also apparent because the viscosity of the myosin–salicylaldehyde complexes becomes non-Newtonian only at higher protein concentrations than 5 mg/ml (Fig. 12). A similar non-Newtonian behaviour of the myosin complexes formed at low ionic strength was observed by JOSEPHS AND HARRINGTON[19]. In the experiment of these authors the reduced viscosity of myosin was observed to decrease with increasing shearing stress whereas in our experiment the reduced viscosity increased with increasing shearing stress. The phenomenon—very rarely observed—indicates that the interaction between the myosin molecules increases with rising shearing stress, and it seems that the structure of the solution becomes more stabilized if the shearing stress is high enough.

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