ACTION OF DINITROPHENYL AMINO ACIDS ON SKELETAL MUSCLE PROTEINS

III.* EFFECT OF PARTIALLY BLOCKING MYOSIN SULPHYDRYL GROUPS ON ABSORPTION OF BIS-DINITROPHENYL-LYSINE; IMPLICATIONS FOR THE STRUCTURE OF MYOSIN WITH MODIFIED ENZYME ACTIVITY

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Summary

Isotherms for the total absorption of bis(2,4-dinitrophenyl)-L-lysine (bis-DNP-lysine) by rabbit skeletal muscle myosin and heavy meromyosin whose sulphydryl groups had been progressively blocked with p-chloromercuribenzoate were measured by equilibrium dialysis in Tris buffers containing potassium chloride.

At high ionic strength ($0 \cdot 5$) the large increase in the adenosinetriphosphatase activity of myosin or heavy meromyosin whose sulphydryl groups had been partially blocked was accompanied by an increase in the amount of bis-DNP-lysine absorbed and there was a maximum on the curve showing the relationship between bis-DNP-lysine absorption and sulphydryl-group blocking. By contrast, at low ionic strength ($0 \cdot 1$) there was no such maximum for aggregated myosin or for monomeric heavy meromyosin after partial blocking of sulphydryl groups, and there was no increase in adenosinetriphosphatase activity.

The results for absorption of bis-DNP-lysine at high ionic strength have been interpreted as evidence that partial blocking of sulphydryl groups displaces a relatively large peptide segment in the heavy meromyosin region.

I. Introduction

It has recently been proposed that useful information on structural changes in proteins, especially in muscle proteins such as myosin which are not always amenable to other methods, may be derived from a study of the absorption isotherms of compounds like bis(2,4-dinitrophenyl)-L-lysine (bis-DNP-lysine) (Burley, Jackson, and Robertson 1967). Thus, the structural change suspected from other evidence to occur on *completely* blocking the sulphydryl groups of myosin with p-chloromercuribenzoate (pCMB) is revealed as an increase in total absorption of bis-DNP-lysine, and, especially at low temperatures, as a large increase in "strong" absorption, that is, the small component of total absorption that is not readily reversible (Burley and Jackson 1965). The effect on the structure of myosin of *partially* blocking sulphydryl groups is discussed here using evidence from changes in the absorption isotherms of bis-DNP-lysine.

The effect of pCMB on myosin is of interest because it is one of a varied group of substances and treatments that modify the adenosinetriphosphatase activity (Kielley and Bradley 1956). After mild treatment with these modifiers the enzyme activity is considerably enhanced, but after more intensive treatment it is completely inhibited. Such modifiers include other sulphydryl-group reagents, e.g. methyl mercuric hydroxide (Kominz 1961), N-ethylmaleimide (Sreter, Seidel, and Gergely 1966), disulphides (Hartshorne and Morales 1965), and copper ions (Blum 1960); water-miscible liquids, e.g. dioxane (Laidler and Ethier 1953; Tonomura *et al.* 1961), and ethylene glycol (Brahms and Kay 1962); dinitrophenol (Greville and Needham 1955); hydrogen peroxide (Rainford, Hotta, and Morales 1964); X-ray treatment (Szabolcs, Zsindely, and Damjanovich 1964); and, possibly, treatment with trypsin (Verpoorte and Kay 1966; Burley 1968). With those modifiers that have been studied in detail, enhancement of adenosinetriphosphatase activity is observed only under certain specific conditions, e.g. with sulphydryl-group reagents in the pH range $6 \cdot 5$–$8 \cdot 5$, at relatively high temperatures (25 rather than 0°C, Stracher and Chan 1961), and at high salt concentration, although this last condition may apply only to myosin from white skeletal muscle (Sreter, Seidel, and Gergely 1966). In addition, enhancement is observed only with calcium-induced adenosinetriphosphatase activity (EC 3.6.1.3), not with that induced by EDTA or by magnesium ions. It is not observed with related substrates; in fact, with inosinetriphosphate inhibition only is observed as a result of treatment with pCMB (Azuma *et al.* 1962). These observations have usually been interpreted in terms of either specific interactions at a few well-defined sites, or deformation of protein structure (e.g. Levy, Leber, and Ryan 1963; Rainford, Hotta, and Morales 1964; Kominz 1965; Gergely 1966). The results presented here tend to support the view that pCMB enhances adenosinetriphosphatase activity of myosin by a limited deformation of protein structure.

II. Materials and Methods

Preparation of myosin and heavy meromyosin from white skeletal muscles of rabbits, and also composition of buffers and most other reagents have already been described (Burley, Jackson, and Robertson 1967, 1968).

(a) Treatment of Sulphydryl Groups

Blocking of sulphydryl groups in myosin and heavy meromyosin was carried out essentially by the method described previously (Burley and Jackson 1965): a calculated amount of standard pCMB was added to the appropriate volume of myosin solution ($0 \cdot 1 \%$ v/w) and the mixture allowed to stand for at least 30 min at 5°C before further treatment. In some experiments protein sulphydryl groups were determined by Ellman's (1959) method. Most samples of myosin contained $8 \cdot 0$ moles of sulphydryl groups per 10^5 g, and heavy meromyosin contained $9 \cdot 5$ moles per 10^5 g.

(b) Absorption of Bis-DNP-lysine

With the difference that Tris buffers were used instead of phosphate buffers, absorption of bis-DNP-lysine was estimated as already described (Burley, Jackson, and Robertson 1968). In brief, at *high ionic strength* total absorption was estimated by equilibrium dialysis, equilibration being for 60–70 hr at 5°C and for 20–30 hr at 20°C. As these measurements were restricted to comparison of samples under similar conditions, the differences already reported between different buffers and different methods of measurement could be ignored. At *low ionic strength* total

absorption was measured after myosin plus absorbed reagent had been removed by brief centrifuging, the reaction time being 20 hr. "Strong" absorption was measured by dialysis (Burley, Jackson, and Robertson 1967).

(c) Measurement of Adenosinetriphosphatase Activity

Enzyme activity of myosin and heavy meromyosin solutions or suspensions was estimated from the rate of liberation of inorganic phosphate from ATP, using an adaptation of conventional methods as described below.

To two or more 2·0-ml samples of the protein in Tris buffer (0·1% w/v), a small volume (50 μl) of ATP solution (0·10M ATP, 0·05M calcium chloride, pH about 6·8) was added, the final pH being 7·0. After reaction at 20°C for periods of up to 10 min for untreated myosin, 1 ml of trichloracetic acid (15% w/v) was added to stop reaction. Identical samples to which the ATP was added after the trichloracetic acid served as controls. The precipitated protein was removed from each sample by centrifuging at 2000 revolutions/min for 15 min. Inorganic phosphate in the supernatant solutions was then estimated by Allen's (1940) method, enzyme activity being expressed as μmoles liberated per milligram of protein per minute.

III. RESULTS

(a) Experiments on Myosin at High Ionic Strength

Figure 1 shows the effect on the total absorption of bis-DNP-lysine at 5°C, ionic strength 0·5, pH 7, of progressively blocking the sulphydryl groups of myosin
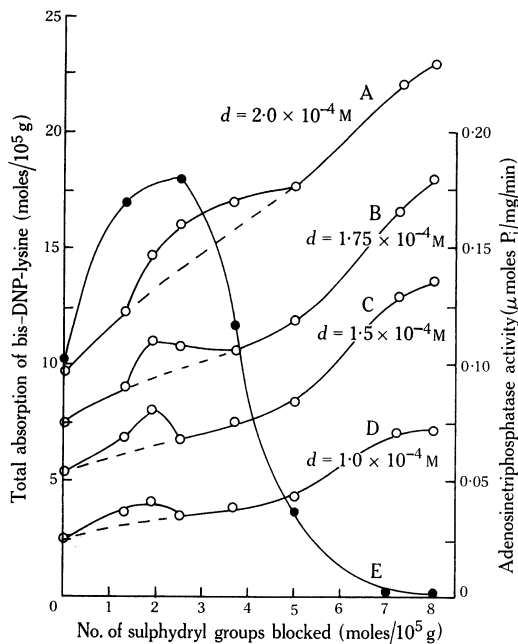

Fig. 1.—Relationship between sulphydryl-group blocking and absorption of bis-DNP-lysine at various equilibrium concentrations d (curves A–D, left hand scale), or adenosine-triphosphatase activity (curve E, right hand scale), both for myosin from one preparation. Absorption measurements were done by equilibrium dialysis at 5°C, ionic strength 0·5, in Tris buffer standardized to pH 7·00 at 20°C, the myosin concentration being 0·1%, v/w. Enzyme activities were measured at 20°C. P_i, inorganic phosphate.

with pCMB. Curves A, B, C, and D refer to different equilibrium concentrations of bis-DNP-lysine. The points on these curves were derived from a series of absorption isotherms, four of which are shown in Figure 2. One preparation of myosin was used for these measurements which were done at the same time.

Curve E in Figure 1 shows the well-known effect of blocking sulphydryl groups on the adenosinetriphosphatase activity of myosin. The enzyme activities were
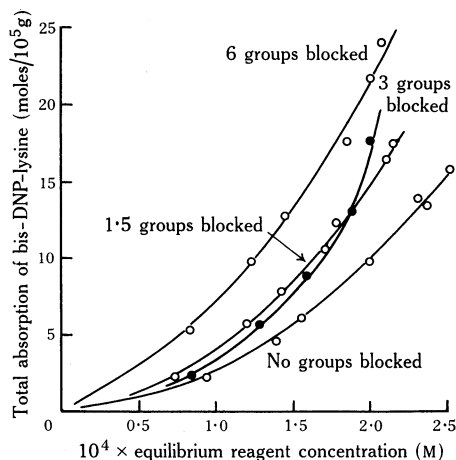

Fig. 2.—Examples of isotherms at 5°C for absorption of bis-DNP-lysine by myosin with different degrees of sulphydryl-group blocking (indicated on curves). These and similar isotherms were used to derive curves A–D in Figure 1. Conditions for measurements were as for Figure 1.
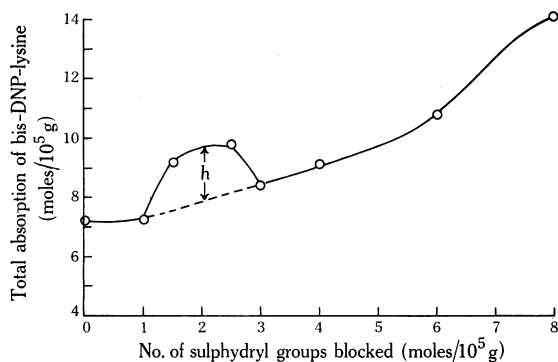

Fig. 3.—Effect of sulphydryl-group blocking on absorption of bis-DNP-lysine by myosin at 20°C, pH 7·0. Ionic strength 0·5, myosin concentration 0·1%, equilibrium concentration of reagent $1·6 \times 10^{-4}$M.
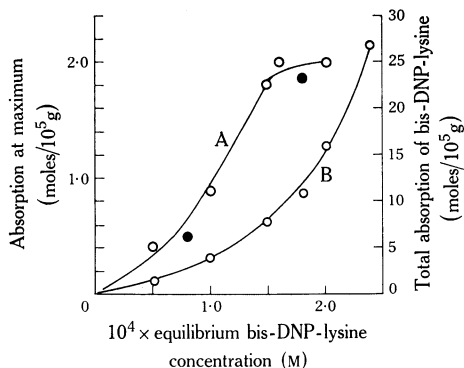

Fig. 4.—Absorption of bis-DNP-lysine by myosin with two sulphydryl groups blocked per 10^5 g; conditions as for Figure 1. Curve A (left hand scale): excess absorption corresponding to the "small maximum", i.e. h in Figure 3. ● Measurements at 20°C. Curve B (right hand scale): total absorption of bis-DNP-lysine at the same equilibrium concentration.

measured at 20°C although, as already stated, bis-DNP-lysine absorption was measured at 5°C. Figure 3, which refers to another myosin preparation, shows that

similar results were also obtained when absorption of bis-DNP-lysine was measured at 20°C.

The data in Figures 1 and 3 reveal a small maximum in the absorption of bis-DNP-lysine corresponding approximately to the maximum in adenosinetriphosphatase activity. The relationship between the height of this maximum, estimated as h in Figure 3, and the equilibrium concentration of bis-DNP-lysine is shown in curve A, Figure 4, which also gives corresponding total absorptions (curve B). Curve A has a sigmoid shape and indicates that the additional absorption responsible for the maxima in Figures 1 and 3 reaches a definite limit, unlike the total absorption, which increases almost exponentially in the same concentration range.
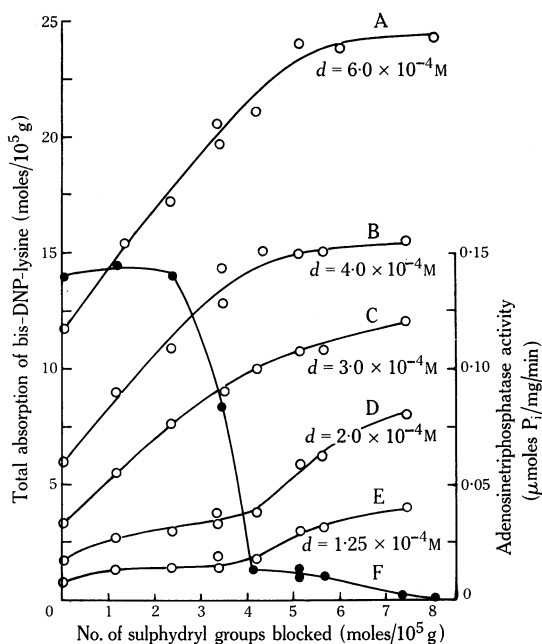

Fig. 5.—Relationship between sulphydryl-group blocking of myosin at ionic strength $0 \cdot 1$ and bis-DNP-lysine absorption at various equilibrium concentrations d (curves A–E, left hand scale) or adenosinetriphosphatase activity (curve F, right hand scale). The absorption curves were derived from isotherms measured at 5°C; other conditions as described for Figure 1.

(b) Experiments on Myosin at Low Ionic Strength

Figure 5, which is analogous to Figure 1, refers to myosin aggregates at ionic strength $0 \cdot 1$. Curves A–E show absorption of bis-DNP-lysine at 5°C. At 20°C curves of the same shape were obtained. Curve F shows the effect of blocking sulphydryl groups on the adenosinetriphosphatase activity of aggregated myosin that had not been treated with bis-DNP-lysine.

From Figure 5 it is clear that at low ionic strength the absorption curves have no maxima corresponding to partial blocking of sulphydryl groups. At low

bis-DNP-lysine concentrations (curves *D* and *E*), however, there is a definite rise in absorption corresponding to complete inhibition of enzyme activity.

(c) *Experiments on Heavy Meromyosin*

Figure 6 shows the effect of blocking sulphydryl groups on the bis-DNP-lysine absorption at one concentration of heavy meromyosin, and on corresponding adenosinetriphosphatase activities, at high and low ionic strength, pH 7·0. The curves in Figure 6 are similar in shape to the corresponding curves in Figures 1 and 5, although there are quantitative differences.
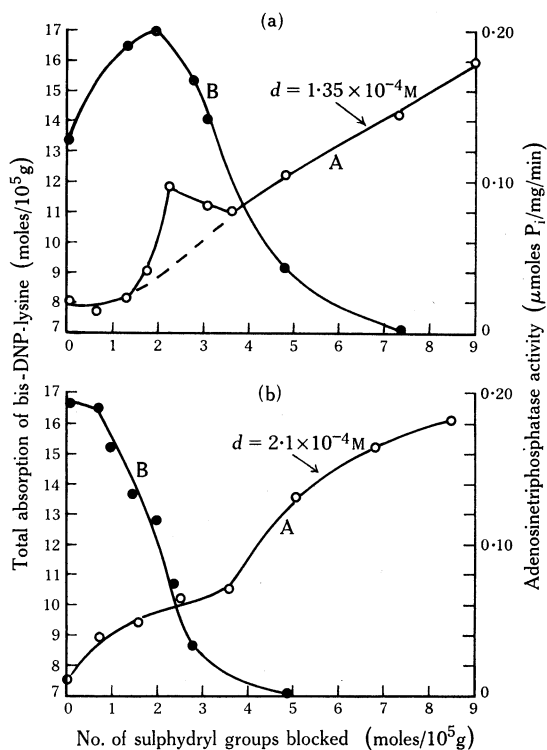

Fig. 6.—Effect of sulphydryl-group blocking on bis-DNP-lysine absorption (*A*, left hand scales, measurements at pH 7, 5°C, and equilibrium concentrations shown) and adenosinetriphosphatase activity (*B*, right hand scales, measurements at 20°C, pH 7) of heavy meromyosin at ionic strengths of 0·5 (*a*) and 0·1 (*b*).

IV. DISCUSSION

The above experiments reveal that at high salt concentration there is a small but unmistakable maximum on the curve relating total absorption of bis-DNP-lysine to the degree of sulphydryl-group blocking (Fig. 1), whereas at low salt concentration such maxima are not observed (Fig. 5). Experiments with heavy meromyosin show the same dependence on salt concentration (Fig. 6). It has already been reported that

what is now referred to as "strong" absorption of bis-DNP-lysine is not noticeable at high ionic strength and low temperature until all the myosin sulphydryl groups have been blocked (Burley and Jackson 1965), so the additional bis-DNP-lysine absorbed after partial blocking would be expected to be weakly bound. The experiments on heavy meromyosin were not complicated by a change in the state of aggregation of the protein, because untreated heavy meromyosin remained monomeric under all the conditions used, although myosin formed large aggregates at low ionic strength (Huxley 1963).

The maxima in the absorption of bis-DNP-lysine may at present best be interpreted as evidence that at high ionic strength the blocking of a small proportion of the sulphydryl groups in the heavy meromyosin region of the myosin molecule leads to a change, probably in protein configuration, to give a structure with a slightly higher absorptive capacity. With both myosin and heavy meromyosin the well-known maximum in the adenosinetriphosphatase activity as a result of partial sulphydryl-group blocking (Kielley and Bradley 1956) occurs in the same region as that for absorption of bis-DNP-lysine (Figs. 1 and 6). The maxima do not coincide, but the correspondence is sufficiently close to suggest that the increased absorption of bis-DNP-lysine is another consequence of the change that leads to enhancement of enzyme activity. Accordingly, it is tentatively proposed that increased enzyme activity and increased binding of bis-DNP-lysine are both a consequence of the displacement of susceptible peptide segments following reaction of certain sulphydryl groups with pCMB. These peptide segments would each need either to possess or be adjacent to several sulphydryl groups such that when about half are blocked with pCMB enzyme activity and absorption of bis-DNP-lysine both increase, but when all have been blocked enzyme activity and absorption of bis-DNP-lysine decrease. Another structural displacement may be responsible for this second change; alternatively, the increased negative charge due to the additional carboxyl groups of pCMB may tend to repel ions of ATP and bis-DNP-lysine. The proposed susceptible peptide segments would be expected to be relatively large. Their size cannot, however, be estimated reliably without further information on the number of enzyme active centres per myosin molecule, and without information on the nature of the atomic arrangement responsible for absorption of bis-DNP-lysine. If the following assumptions are made: (1) that there are two active centres per myosin (Slayter and Lowey 1967), (2) that about five bis-DNP-lysine molecules or ions are absorbed per active centre (based on calculations from Fig. 4 using 5×10^5 g as the molecular weight of myosin), though not necessarily in the vicinity of the centre, and (3) that three or four amino-acid residues from each peptide segment contribute to the absorption of each bis-DNP-lysine; then it may be deduced that an approximate minimum size for each peptide segment would be 15–20 amino-acid residues.

This explanation, which assumes that the deformation of a large segment of a peptide chain exerts limited control on the enzyme activity of myosin, may also help to account for the similar effects of a variety of modifiers with quite different chemical properties, examples of which have been given in Section I. Their common property would be the ability to displace or remove the proposed peptide segments in a similar though not necessarily identical manner. This should be easier if each of the peptide segments possessed a variety of different amino-acid residues with affinities for many

different kinds of reagent. It is more difficult to imagine how the displacement of the proposed peptide segments controls the adenosinetriphosphatase activity, especially as there is evidence that the sulphydryl groups whose blocking is responsible for enhancement of activity may be at a considerable distance from the active centre (Trotta, Dreizen, and Stracher 1968). Possibly the proposed peptide segments control the access of ATP to the active centre.

Figure 1 also suggests that superimposed on the changes assumed to be responsible for both enzyme activation and the small maximum in absorption of bis-DNP-lysine are other changes that become prominent as the blocking of sulphydryl groups approaches completion. These changes lead to complete inhibition of enzyme activity, to a large increase in absorption of bis-DNP-lysine, and to the phenomenon referred to as strong absorption. The complete removal of sulphydryl groups appears, from the large amounts of bis-DNP-lysine absorbed, to involve extensive loosening of peptide chains in the heavy meromyosin region (Burley, Jackson, and Robertson 1967), rather than strictly limited displacements of the sort proposed above for partial blocking.

V. ACKNOWLEDGMENTS

VI. REFERENCES

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