Myosin Modification as Studied by Spin Labeling*

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ABSTRACT: Nitroxide-type spin labels with reactivity toward sulfhydryl groups are covalently attached to myosin, and exhibit both strongly and weakly immobilized-type spectra. Attachment of even 1 mole of label/mole of myosin "modifies," *i.e.*, activates the Ca^{2+}-ATPase of the enzyme. Nevertheless, if the labeled myosin is then maximally activated by adding *p*-mercuribenzoate, the resulting spectrum, relative to the original spectrum, indicates that the number of weakly immobilized labels has increased. If further *p*-mercuribenzoate is added until the Ca^{2+}-ATPase is destroyed, the resulting spectrum, relative to the original spectrum, indicates a very marked increase in the number of weakly immobilized labels.

It is concluded that activation is associated with a slight, and inhibition with an extensive, conformational change of the myosin molecule.

It was surmised by Rainford *et al.* (1964) and by Sekine and Kielley (1964) that progressive "modification" of myosin Ca^{2+}-ATPase activity (in both cases through progressive blocking of SH groups) was accompanied by enzyme conformational changes. Nonkinetic evidence for this view was provided by Duke *et al.* (1966; see also Cheung and Morales, 1969), observing fluorescence enhancement of a myosin-bound "*hydrophobic*" dye in response to SH reagents, chelators, and alkalinity. In the present paper we study the same phenomenon by observing the electron paramagnetic resonance spectra of a myosin-bound SH-directed spin label, *N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide (Ogawa and McConnell, 1967; Hamilton and McConnell, 1968), hereafter called "X."[1] Yet a third substantiation, simultaneous with this work, is being provided by Cheung and Morales (1969), who has observed the myosin–dye energy-transfer efficiency in response to several modifiers. The significance of these variously detected conformational changes of myosin lies in the possibility that such changes are involved when this enzyme is "modified" by actin, in the course of thrust generation.

Experimental Procedures

Preparations of myosin were made according to Tonomura *et al.* (1966), and initially all had enzymatic activities near 5 μmoles of P_i g^{-1} sec^{-1}, in solvent 0.6 M KCl, 0.05 M Tris, and 10 mM $CaCl_2$, and at pH 8.00, 25°. Modification and labeling reactions were usually initiated within 1 or 2 days of preparation. The electron paramagnetic resonance spectra were usually recorded within 1 week of preparation, and never after 2 weeks.

Baker "Analyzed" KCl was used without further purification; α-IAA, obtained from Sigma, was recrystallized once from water, and the recovered white crystals were stored in the dark at 0° until needed; PMB was obtained from CalBiochem, and was recrystallized three times, according to Boyer (1954). X was synthesized and purified according to Ogawa and McConnell (1967).

ATPase activities were measured by sequential P_i analysis, using a slight variant of the Fiske–Subbarow method; protein concentrations were measured by a variant of the Folin–Cicalteau method (Gellert *et al.*, 1959); SH contents were measured by a variant of Boyer's method (Rainford *et al.*, 1964).

Spin-labeled myosin was prepared by incubating an equimolar solution of protein and reagent for 48–60 hr at 0°; the electron paramagnetic resonance spectrum indicated that shorter incubations produced insufficient labeling, while incubations up to 272 hr did not lead to greater labeling. Following incubation the reaction was terminated by dialysis at 0°, against 0.6 M KCl–0.05 M Tris (pH 8.0), for 24 hr with five changes; dialysis removes unreacted label.

When modifying spin-labeled myosin with PMB the proper aliquot of the concentrated PMB solution was mixed with the protein for at least 24 hr at 0°; such samples were not dialyzed further, and spectra were always recorded within 96 hr after PMB addition. The amount of PMB required for maximum activation of ATPase was taken to be 45% of the free SH content of myosin, about 11–14 moles of PMB/mole of myosin. When IAA was the modifier the procedure was analogous except that the time for modification was lengthened to 72 hr. Modified (by either PMB or IAA) myosin was spin labeled by analogous techniques; in some cases, after the dialysis step, some samples were still further modified by additional PMB, for at least 24 hr at 0°.

Care was taken during all preparative steps to prevent aggregation of the sample while maintaining the protein concentration high (2% or more). When the labeling ratio is 1 mole of label/mole of myosin it is essential to use high protein concentration in order to assure adequate signal/noise ratios in the resulting spectra.

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[1] Abbreviations used are: X, *N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide; IAA, iodoacetamide; PMB, *p*-mercuribenzoate.

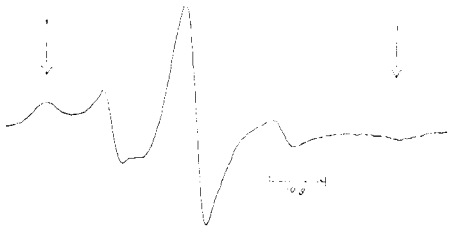

FIGURE 1: Spectrum of X spin-labeled myosin in 0.6 M KCl (pH 8.0). Labeling ratio: 0.2 mole of X to 10^5 g of myosin. Vertical arrows indicate bands arising from "strongly immobilized" spin label. Here and elsewhere the magnetic field increases to the right.

Spectra were recorded at room temperature, in a Varian Associates Model E-3 electron paramagnetic resonance spectrometer operating at 9500 Mc. When necessary several repeated spectra were averaged in order to improve the signal/noise ratio.

Results

The electron paramagnetic resonance spectrum of myosin spin labeled (0.2 mole/10^5 g of myosin) with X is shown in Figure 1; 10 mM $CaCl_2$ has no effect on this spectrum. The sharp, three-line hyperfine pattern arises from attached X molecules with considerable mobility or rotational freedom ("weakly immobilized" or "loose" labels); this component of the spectrum indicates the flexibility of the side chains to which label is attached.[2] The broad lines indicated by arrows on Figure 1 arise from attached X molecules whose half-time of rotation relative to the myosin is at least as long as the correlation time for the tumbling of the myosin ("strongly immobilized" or "tight" labels). These broad lines cannot be prevented by limited SH blocking prior to spin labeling (see below).

When myosin is treated with PMB in equimolar proportion (0.2 mole of PMB/10^5 g of myosin) the ATPase activity is elevated very little, and when it is treated with 12 moles of PMB/mole of myosin the ATPase activity is elevated three- or fourfold; therefore, between the two levels of treatment there is a large increase in degree of modification. By contrast, equimolar treatment with IAA or X more than doubles the ATPase activity (presumably these reagents are more specific for activating groups than is PMB); if such enzyme is then treated with PMB until there are 12 moles of blocked SH/mole of myosin the activity increases only 25% (Tables I and II). Nevertheless, comparison of "base" and "activated" spectra shows that in the activation process the environments of some labels have been changed (Figure 2); relative to the base spectrum there is a slight sharpening of the three central peaks, *i.e.*, the number of loose or weakly immobilized spin labels has been slightly increased. The ratio of the height of the first peak, representing strongly immobilized spin labels, to that of the second peak, representing weakly immobilized spin labels, decreases from 0.60 to 0.56 upon activation with PMB. Although the foregoing difference between spectra is small, it is reproducible; the difference persists, and is even enhanced (Figure 3), if the solvent is reduced in ionic strength (from 0.6 M KCl to 0.06 M KCl).

Overmodification of spin-labeled myosin by reaction with excess PMB results in progressive inhibition, and eventually in total loss of ATPase activity. During the initial stages of inhibition the electron paramagnetic resonance spectrum sharpens only slightly; at or near the stage of total inhibition (*e.g.*, 35 moles of PMB/mole of myosin), however, the spectrum sharpens markedly (Figure 4). Very similar effects were observed when myosin which had been maximally accelerated with PMB prior to labeling with X (1 mole/mole of myosin) was treated with excess PMB.

The *sequence* in which the same amounts of reagents are added to myosin appears to make a difference in enzymatic properties and in electron paramagnetic resonance spectra. Thus, if to 1 mole of myosin 1 mole of X is added, then 12 moles of PMB, or if 12 moles of PMB are added, then 1 mole of X, the spectra of the results are different (Figure 5) and the enzymatic activities are different (Table II). When myosin is

TABLE I: Ca^{2+}-ATPase of Modified[a] Myosin.

Modifier	ATPase (μmoles of P_i/g sec)
1. None (control)	4.06
2. Spin label	12.8
3. None (control)	4.68
4. PMB	5.05
5. IAA	12.4

[a] The degree of modification in each case is 0.2 M/10^5 g. Conditions: 0.6 N KCl, 0.05 M Tris (pH 8.0), 25°, and 10^{-2} M $CaCl_2$.

TABLE II: Ca^{2+}-ATPase of Spin-Labeled Modified Myosins.[a]

Modifiers	ATPase (μmoles of P_i/g sec)
1. None (control)	4.06
2. Spin label + PMB	15.8
3. Spin label + IAA	39.4
4. PMB + spin label	8.10
5. IAA + spin label	25.0

[a] Conditions: 0.6 N KCl, 10^{-2} M $CaCl_2$, 0.05 M Tris (pH 8.0), and 25°. Samples 2 and 3 were spin labeled prior to modification. Samples 4 and 5 were modified prior to spin labeling. Degrees of modification: spin label at 0.2 mole/10^5 g; PMB and IAA at 2.4 M/10^5 g.

[2] Contributions to the weakly immobilized region of the spectrum could conceivably arise from the labeling of ϵ-NH_2 groups of lysyl residues, as well as from labeling of sulfhydryl (which is assumed here). Utilizing X, Ogawa and McConnell (1967) (unpublished) observed the appearance of weakly immobilized bands when investigating hemoglobin whose SH groups had been previously blocked. Furthermore, Stone *et al.* (1965) have shown that a spin label with isocyanate reactivity moves remarkably freely when combined with poly-L-lysine, whether the polypeptide is helical or randomly coiled.

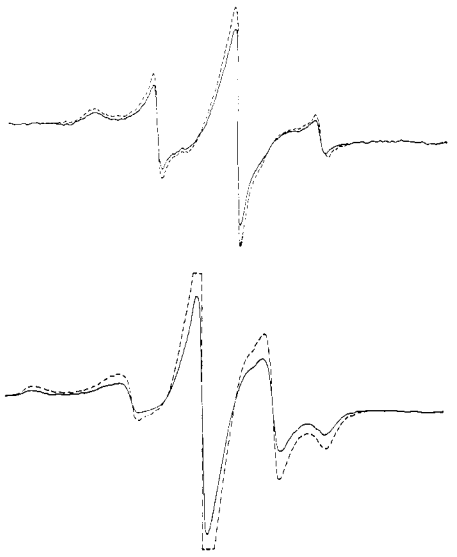

FIGURE 2: Comparison of the "base" spectrum with the spectrum of PMB-activated myosin, both in 0.6 M KCl (pH 8.0). Solid line: myosin incubated in the proportion 0.2 mole of X to 10^5 g of myosin. Dotted line: similarly labeled myosin then treated with PMB in the proportion 2.4 moles of PMB to 10^5 g of myosin.

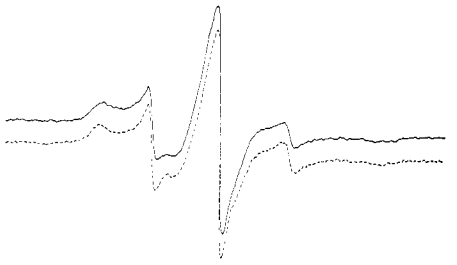

FIGURE 3: Comparison of the "base" spectrum with the spectrum of PMB-activated myosin, both in 0.06 M KCl (pH 8.0). Each of the samples of Figure 2 has been changed from 0.6 to 0.06 M KCl.

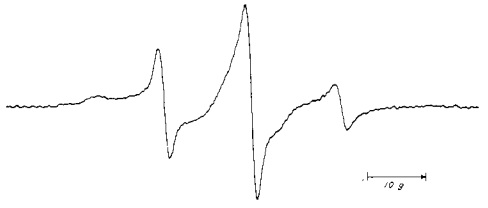

FIGURE 4: Spectrum of X spin-labeled myosin totally inactivated by excess PMB, in 0.6 M KCl. Myosin incubated in the proportion 0.2 mole of X to 10^5 g of myosin, then reacted in the proportion 7 moles of PMB to 10^5 g of myosin.

first activated with PMB or IAA, then labeled with X (latter sequence), the resulting activity is less, and the spectrum is broader, than in the product of the opposite sequence. Modification must therefore direct label to sites other than those to which it would go initially; this effect occurs when modification is as slight as 1 mole/mole of myosin.

When myosin is labeled with X, then is further modified

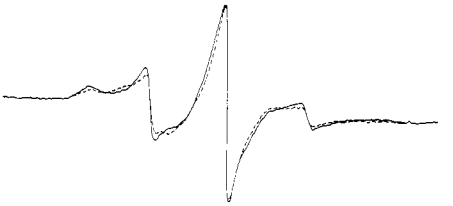

FIGURE 5: Spectral comparison in 0.6 M KCl of myosin which has been X labeled, then PMB activated, with myosin which has been PMB activated, then labeled. Solid line: myosin labeled in the proportion 0.2 mole of X to 10^5 g of myosin, then reacted in the proportion 2.4 moles of PMB to 10^5 g of myosin. Dotted line: myosin treated in the reverse sequence.

with IAA (rather than PMB), the further increment in ATPase is much greater than with PMB (Table II), but the electron paramagnetic resonance spectrum is not significantly different from base.

Discussion

The spectra resulting merely from labeling myosin with X give independent evidence for the heterogeneity of SH groups, since some attached labels are clearly weakly immobilized and some are strongly immobilized. Another by-product of this study is knowledge that molecules with iodoacetamide reactivity are much more specific than is PMB for those SH groups whose reaction results in activation of myosin Ca^{2+}-ATPase. This has both advantages and disadvantages *vis-à-vis* the main object of the study. The advantage is that such changes as may result from the activation process are not apt to be confused by the concomitant reaction of SH groups involved in the ATPase-"killing" process. The disadvantage is that activation proceeds a considerable distance simply as a result of labeling, so that subsequent reaction of labeled myosin with PMB leads only to a relatively small ATPase increment.

The increase in number of weakly immobilized labels, hence the presumed conformational change, resulting from activating spin-labeled myosin with PMB is small. It is, however, completely consistent with the result of fluorescence probing (Duke *et al.*, 1966). Since the increase resulting from activating with IAA instead of PMB was undetectable, it could be thought that such spectral change as we did observe with PMB was due to slight parallel reaction with "kill" groups. This, however, is unlikely since Duke *et al.* (1966) observed slight increases in myosin-bound dye fluorescence upon activation with EDTA which does not "kill" at any concentration, and Cheung and Morales (1969) has observed a slight increase in energy-transfer efficiency on going from native to fully IAA-activated enzyme. When spin-labeled myosin is treated with PMB so as to abolish ATPase activity there is a very large increase in weakly immobilized labels, much as there is a large increase in bound dye fluorescence or in energy transfer to bound dyes. The presumably large conformational change suggested by these phenomena is not a typical "denaturation"; it is barely perceptible in the optical rotatory dispersion spectrum, and it can be largely reversed; very possibly it reflects a partial unravelling of the two "heads" of myosin. The foregoing experiments on varying the sequence in which label and reagent are added were originally undertaken with a

more ambitious objective, but they do show that such sequencing provides a crude means whereby label can be directed to qualitatively different SH groups. That the final properties (ATPase activity and spectrum) depend upon sequence is suggestive, but at present nothing can be made of it, since as yet we have no means of ascertaining that the same number of moles of a reagent became attached in the two sequences.

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