Graphical Evaluation of Alkylation of Myosin's SH1 and SH2: The *N*-Phenylmaleimide Reaction

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ABSTRACT Previous assertions about the effect of alkylation of SH1 and SH2 on the myosin high-salt calcium and EDTA ATPases have been summarized, and a simple procedure for obtaining the fractional labeling of SH1 and SH2 after treatment of myosin with alkylating agents has been derived. A simple graphical procedure for illustrating the degree of preference of a particular alkylating agent for SH1 over SH2 has also been developed. The procedures we developed were validated by applying them to two previously studied compounds, 4-(2-iodoacetamido)-TEMPO and 2,4-dinitrofluorobenzine, and then were used to determine a procedure for maximizing the extent of labeling of SH1 alone by *N*-phenylmaleimide, a compound not previously studied in this manner. It was found that ~80% of the SH1 sites could be alkylated without significant alkylation of SH2.

INTRODUCTION

Although a number of proteins are involved in force generation by skeletal muscle, myosin and actin are the most abundant and presumably the most important. The ability of actin and myosin to generate force in muscle fibers and to hydrolyze ATP rapidly under physiological conditions is exquisitely sensitive to modification of myosin's two most reactive sulfhydryls, SH1 (Cys-707) and SH2 (Cys-697); when both of these sulfhydryls are modified, a muscle fiber will not produce force and actomyosin will not hydrolyze ATP (Yamaguchi and Sekine, 1966; Reisler et al., 1974; Chaen et al., 1986; Ehrlich et al., 1995).

Previously we showed that in the presence of MgATP, *N*-phenylmaleimide (NPM) links to both SH1 and SH2 of myosin and locks the myosin cross-bridge in a weakly binding configuration (Barnett et al., 1992; Ehrlich et al., 1995). As a continuation of those studies, we would like to know whether the locking effect is due to the linkage at SH1 or at SH2, or whether both sulfhydryls must be alkylated for the effect to occur. One way of exploring this question is to look at the behavior of cross-bridges with NPM linked solely to SH1. Root et al. (1991) and Roopnarine and Thomas (1994) have shown that reacting myosin in the presence of Mg_2PP_i rather than MgATP favors labeling more specifically at SH1 compared to SH2. Unfortunately, it is thought that maleimide compounds like MSL are less specific for SH1 than iodoacetamide compounds such as 4-(2-iodoacetamido)-2, 2, 6, 6-Tetramethyl-1-piperidine-N-oxyl (IASL) or iodoacetic acid (Reisler et al., 1977). Thus it is not clear how large a fraction of SH2 sites would become alkylated if one were to use the technique of Roopnarine et al. to try to label myosin at SH1 with NPM. What is needed is a way of quantifying the relative amount of SH1 and SH2 labeling that would be generated.

Previously we used analytical high-performance liquid chromatography and peptide sequencing to determine quantitatively the sites of NPM reactivity (Ehrlich et al., 1995). That procedure, however, was difficult and time consuming. Of the many studies of alkylation of SH1 and SH2, relatively few have used peptide analysis to determine the site of reaction; most have relied upon ATPase data to provide information about the site of reactivity.

The ATPase activities usually measured to provide information about SH1 and SH2 alkylation are myosin ATPases measured at high salt (0.6 M KCl) to minimize the influence of actin. The two measurements are made 1) in the presence of EDTA and 2) in the presence of 10 mM calcium (Yamaguchi and Sekine, 1966; Reisler et al., 1974; Thomas et al., 1980). Two of the more important observations from these studies are 1) reaction of a myosin head's SH1 sulfhydryl eliminates that head's ability to hydrolyze ATP in the absence of divalent ion (loss of EDTA ATPase activity) and increases its ability to hydrolyze ATP in the presence of divalent ion (increase in calcium ATPase activity), and 2) the subsequent reaction of SH2 eliminates both the EDTA and calcium ATPase activities (Sekine and Keilley, 1964; Yamaguchi and Sekine, 1966). Although these assertions may not apply in all instances (Wiedner et al., 1978), it is thought they generally have wide applicability.

Whereas a simple expression for relating the measured ATPase activities to the fractional alkylation of SH1 already is available (see Roopnarine et al., 1993), so far as we know, no equivalent expression for SH2 has been published. In this paper, we succinctly summarize what is known about the relationship between the EDTA and calcium ATPase activities and SH1 and SH2 modification, and we develop a simple method for relating the high-salt myosin ATPases to the fractional modification of both SH1 and SH2. The approach is illustrated and tested by applying it to two

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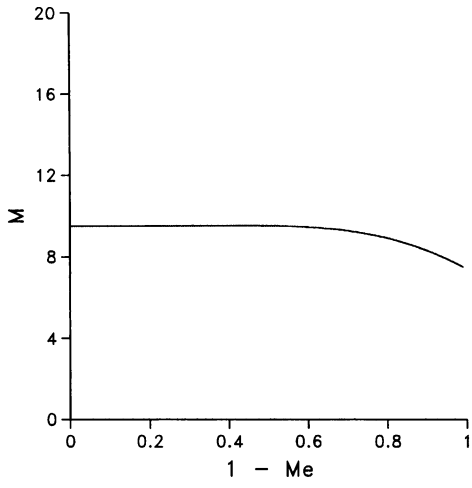

FIGURE 1 Theoretical relationship between M and $1 - Me$. M is defined in text as $(Mc - 1)/(1 - Me)$. $1 - Me$ is a measure of the extent of labeling. When only SH1 is labeled (as is likely with brief labeling), the relationship is flat. With SH2 labeling (as is more likely, the greater the extent of labeling) the relationship curves downward.

previously studied alkylating agents, IASL and FDNB. Using this procedure as a tool for quantifying the fraction of SH2 alkylated, we examine the alkylating agent *N*-phenylmaleimide, and conditions suitable for maximizing the fraction of phenylmaleimide label solely at SH1 are derived.

MATERIALS AND METHODS

Reagents

IASL and NPM were obtained from Aldrich Chemical Company (Milwaukee, WI). 2,4-Dinitrofluorobenzine (FDNB) was obtained from Sigma (St. Louis, MO). NPM was dissolved in dimethylformamide to a final concentration of 10 mM. FDNB was dissolved in dimethylformamide to a final concentration of 50 mM. Both stock solutions were discarded after 30 days. Control experiments showed that the dimethylformamide that was transferred to the solutions with the alkylating agents had no effect. IASL was dissolved in buffer immediately before usage. Dithiothreitol (DTT) was obtained from ICN Biochemicals (Aurora, OH).

Treatment of the fiber bundles

The rabbit psoas muscle bundles were dissected as described by Ehrlich et al. (1995). The sarcolemmas of the bundles were made permeable by incubating the bundle for >1 h at 4°C in skinning solution containing 150 mM KPropionate, 3 mM $Mg(C_2H_3O_2)2$, 3 mM Na_2ATP, 5 mM EGTA, 0.5 mM DTT, and 5 mM KH_2PO_4 (pH 6.8) (Eastwood et al., 1979) and 0.5%(v/v) Triton X-100. After treatment in skinning solution, the bundles were incubated (4°C) for 15 min (2×) in the experimental buffer. For IASL and NPM the buffer contained 40 mM KCl, 2 mM EGTA, 2 mM $MgCl_2$, 2 mM sodium pyrophosphate, and 10 mM imidazole (pH 7.0) (Root et al., 1991). For experiments with FDNB, the buffer contained 130 mM KPropionate, 1 mM EGTA, 5 mM $MgCl_2$, 10 mM K_4PP_i, and 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.0) (Roopnarine and Thomas, 1994). These buffers were chosen to maximize the labeling of SH1 relative to SH2 (Root et al., 1991; Roopnarine and Thomas, 1994). The bundles were treated for different amounts of time by incubation in the experimental buffer containing IASL (0.50 mM), FDNB (0.02 mM), or NPM (0.10 or 0.15 mM). The reaction was terminated by transferring the fiber bundles to fresh buffer that had 1 mM DTT and no reagent (Reisler, 1982).

Assay of ATPase activities

Muscle bundles were converted to myofibrils in 10 mM MOPS (pH 7.0) using a Tissue Tearor homogenizer (Biospec Instruments, Bartlesville, OK) at 5000 rpm. The myofibril concentration was determined by Lowry assay (Lowry et al., 1951). The EDTA and calcium ATPase activities were measured in a buffer containing 5 mM EDTA, 0.6 M KCl, 50 mM MOPS (pH 7.5), or 10 mM $CaCl_2$, 0.6 M KCl, 50 mM MOPS (pH 7.5). The reaction was initiated (25°C) by the addition of 0.1 M ATP to achieve a final concentration of 5 mM, and the production of inorganic phosphate over a 5-min period was measured (Roopnarine et al., 1993; Lanzetta et al., 1979). The amount of protein used in the assays ensured a linear ATPase rate and was either 50 μg (EDTA ATPase) or 100 μg (calcium ATPase) in 1 ml.

Error bars on the figures

In the figures, the error bars show the standard error of the mean (SEM). For directly measured variables, such as the ATPases, the SEM was calculated in the standard fashion, making the assumption that the measured variables were normally distributed about a mean value. For derived variables such as *Me*, *Mc*, *M*, *X*, and *Z* (defined in the next section), the assumption of a normal distribution should not, most properly, be assumed. In this case the SEM of the derived variable was calculated from the SEM

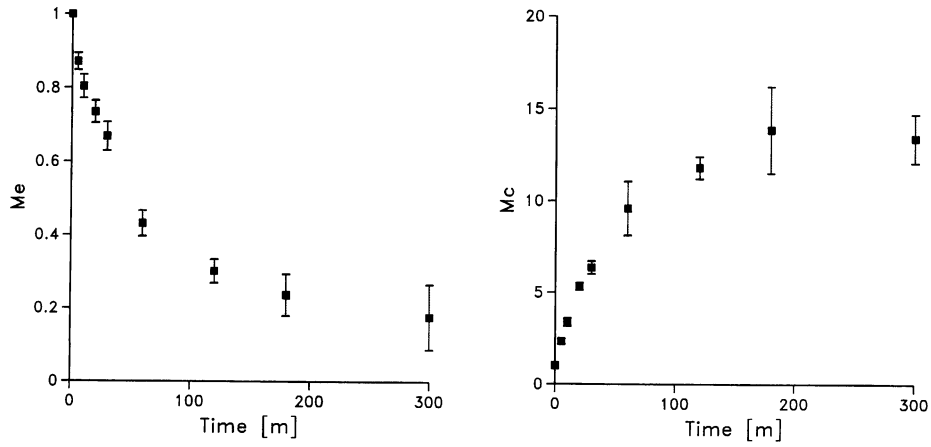

FIGURE 2 Normalized EDTA and calcium ATPase activities (*Me* and *Mc*) as a function of treatment duration when small muscle bundles are treated with 0.5 mM IASL at 5°C.

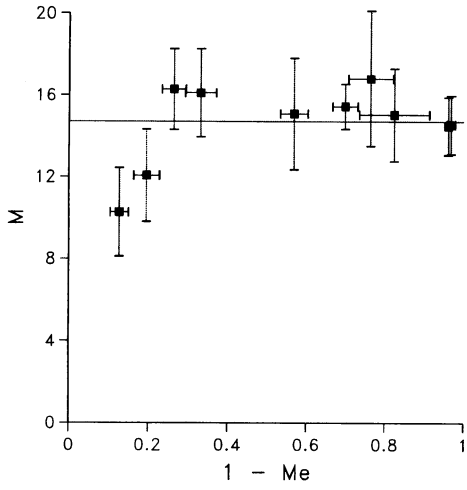

FIGURE 3 The M versus $1 - Me$ relationship for 0.5 mM IASL. The horizontal line shown is the best least-squares fit of a horizontal line through the data points. The y intercept is 14.68 ± 0.57. The fitting was done using a computer program based on Marquardt's compromise (Marquardt, 1963).

of the measured variables using formulae available in many elementary statistics texts. We used formulae from the reference work by Menzel (1960).

RESULTS

Theoretical

For purposes of calculating a muscle fiber's ATPase, one can summarize the numerous references cited in the Introduction as follows:

1. An unmodified myosin head has an EDTA ATPase activity of E and a calcium ATPase activity of C.
2. A head labeled solely at SH1 has an EDTA ATPase activity of zero and a calcium ATPase activity of N^*C. It is not certain whether N is a function of label or is the same for all labels.
3. A head labeled solely at SH2 has an EDTA ATPase activity of zero and a calcium ATPase activity of P^*C.

We refer to the fraction of heads with label solely at SH1 as X and the fraction of heads with label solely at SH2 as Y, and we use the symbol Z to denote the fraction of heads that have label at both SH1 and SH2. Because the reactivity of SH1 is generally much greater than that of SH2, we make, with reasonable accuracy, the simplifying assumption $Y = 0$, i.e., we assume that there are no heads with label solely at SH2. In this case, the fraction of heads with both SH1 and SH2 unmodified is $1 - X - Z$.

Having $Y = 0$ also removes P from the equations. Nonetheless, without knowing the value of N, we cannot determine X and Z from just a single measurement of a fiber's EDTA and calcium ATPase activities. This is because, without knowing N, there are results of two measurements (EDTA and calcium ATPase activities) and three unknowns (N, X, Z). We will show that by measuring a fiber's EDTA and calcium ATPase activities at several different labeling durations, including some that are brief, one can use an assumption made previously by Crowder and Cooke (1984) to determine N. It is then possible to determine both X and Z as a function of labeling duration.

Let Me be the ratio of the EDTA ATPase activity of a treated fiber to that of an untreated fiber, and let Mc be the ratio of the calcium ATPase activity of a treated fiber to that of an untreated fiber. Because the EDTA ATPase activity per head of an untreated fiber is E, that of a treated fiber will be $(1 - X - Z)E$. Therefore, $Me = 1 - X - Z$. Similarly, because the calcium ATPase activity per head of an untreated fiber is C, so that of a treated fiber will be $X \times N \times C + (1 - X - Z)C$, so that $Mc = X \times N + 1 - X - Z = X \times N + Me$.

If $Mc = X \times N + Me$, then $Mc - 1 = X * N + Me - 1$. Dividing both sides of the equation by $1 - Me$, we have

$$\frac{Mc - 1}{1 - Me} = \frac{X^*N}{1 - Me} - 1.$$

If we define $M = (Mc - 1)/(1 - Me)$, we have

$$M = \frac{X \times N}{1 - Me} - 1.$$

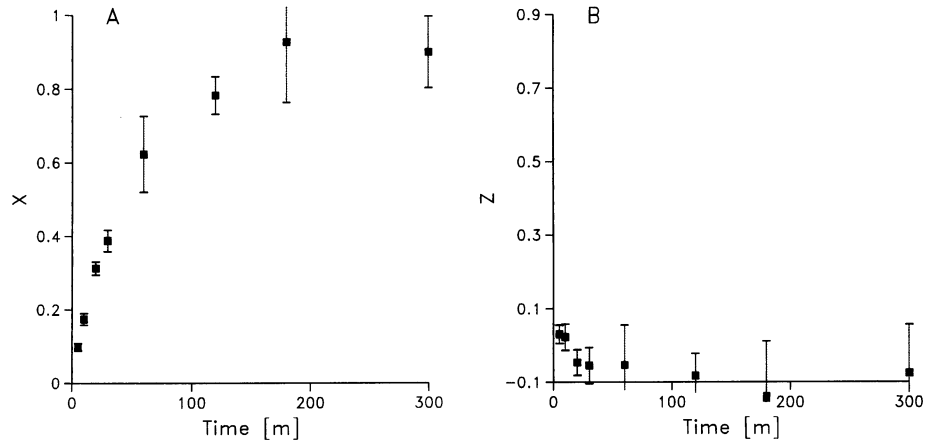

FIGURE 4 (*A*) Fractional labeling of SH1 alone by ASL (X). (*B*) Fraction of myosin heads with SH1 and SH2 labeled (*Z*). Z is close to and indistinguishable from 0. X at 180 m is 0.87 ± 0.15.

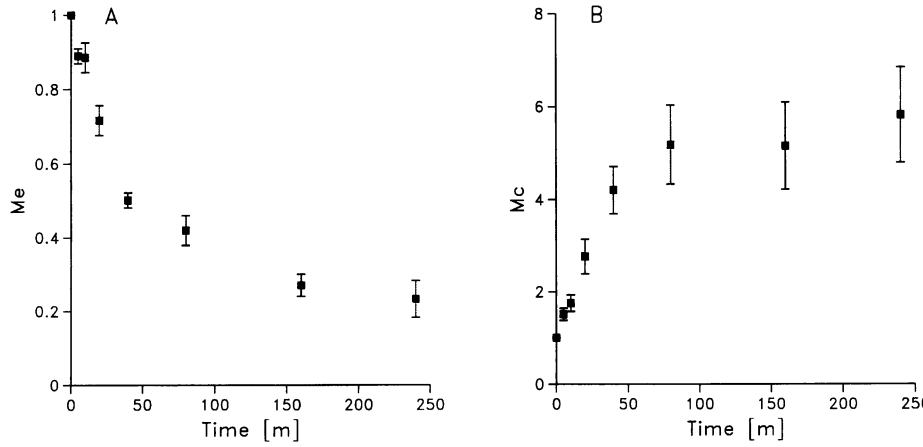

FIGURE 5 Normalized EDTA and calcium ATPase activities (*Me* and *Mc*) as a function of treatment duration when small muscle bundles are treated with 0.02 mM FDNB at 5°C.

We define a variable $K = Z/X$. In that case, $Me = 1 - X - Z$ becomes $Me = 1 - X(1 + K)$. Rearranging gives $1 - Me = X(1 + K)$, so that $M = X \times N/(1 - Me) - 1$ becomes

$$M = N/(1 + K) - 1. \quad (1)$$

Crowder and Cooke pointed out that because of the much greater reactivity of SH1 compared to SH2, K is likely to be zero for brief-duration labeling. It is clear from Eq. 1 that for $K = 0$, $M = N - 1$, and for $K > 0$, $M < N - 1$. It is thus predicted, if all of our assumptions are correct, that if we make a plot of $M(t)$ versus $1 - Me(t)$, where t is treatment duration, it should have the general form shown in Fig. 1.

The M versus $1 - Me$ plot can be a useful tool for evaluating the effects of a given label. To the extent that labeling occurs only at SH1, the M versus $1 - Me$ plot should be a horizontal line. If SH2 labeling were to occur with longer labeling times, the relationship would be concave downward. In addition, it is straightforward to work backward from an M versus $1 - Me$ curve and obtain X and Z as functions of labeling duration. This may be done in a straightforward manner by sequentially calculating

$$N = 1 + M(0) \quad (2)$$

$$X(t) = (Mc(t) - Me(t))/N \quad (3)$$

$$Z(t) = 1 - Me(t) - X(t), \quad (4)$$

where $M(0)$ is the limiting value of M for brief-duration labeling and t is treatment time.

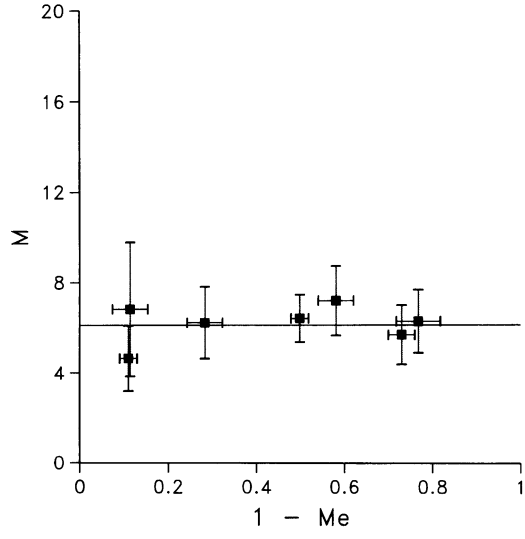

FIGURE 6 The M versus $1 - Me$ relationship for 0.02 mM FDNB. The horizontal line shown is the best least-squares fit of a horizontal line through the data points. The y intercept is 6.1 ± 0.54. The fitting was done using a computer program based on Marquardt's compromise (Marquardt, 1963).

Experimental

IASL and FDNB

Before applying the graphical technique to a totally unknown compound, we applied it to two compounds whose behavior was reasonably well understood from previous work. Fig. 2 shows the summarized data from six experiments on muscle bundles treated with 0.5 mM IASL. The relative EDTA ATPase activity (*Me*) of myosin decreased with increasing IASL modification, and the calcium ATPase activity (*Mc*) increased about 15-fold after 5 h of treatment. With further treatment (up to 46 h), *Me* approached zero (0.05 ± 0.03), and *Mc* remained about the same (15.08 ± 1.38). The calculated M versus $1 - Me$ relationship is shown in Fig. 3. It is clear from the data that M has a similar value no matter what modifying level is achieved. The fact that the M versus $1 - Me$ curve is a horizontal line indicates that under the conditions employed in this paper, IASL reacts specifically with SH1, with very little SH2 reaction. This conclusion also may be drawn from the observation that *Mc* increases monotonically and then levels off and does not decrease with extended treatment. The preference of IASL for SH1 is consistent with previous studies (Seidel et al., 1970; Thomas et al., 1980), and the ability of the graphical method to readily indicate this

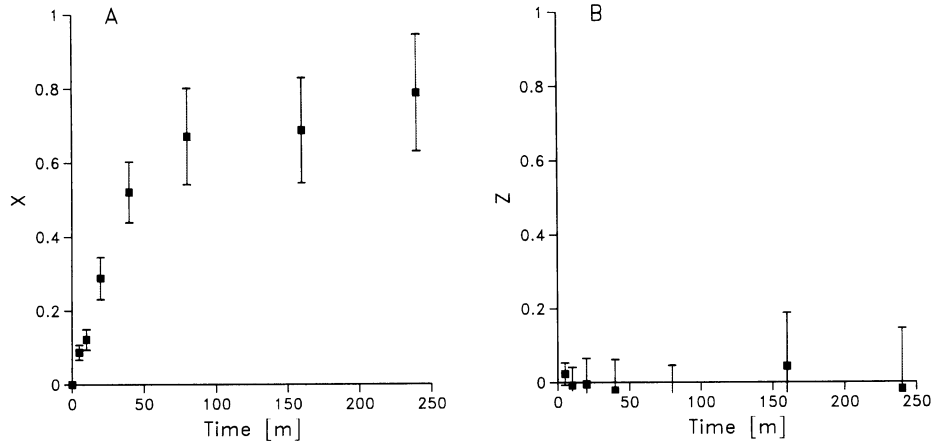

FIGURE 7 (*A*) Fractional labeling of SH1 alone by dinitrophenyl (*X*). (*B*) Fraction of myosin heads with SH1 and SH2 labeled (*Z*). *Z* is close to and indistinguishable from 0.

demonstrates its usefulness for evaluating the specificity of modification of SH1 and SH2.

The graphical method also yields quantitative information on the population of SH1-modified myosin and SH1,SH2-modified myosin. From the data of Fig. 3, $M \approx 15$. According to Eq. 2, $N \approx 16$. The fraction of SH1-modified heads at different treatment times is calculated from Eq. 3 and shown in Fig. 4 *A*. The fraction of SH1,SH2-modified heads is calculated from Eq. 4 and shown in Fig. 4 *B*. Clearly, rather complete labeling of SH1 is achieved and the fraction of SH1,SH2-modified heads is very small.

FDNB, another compound known to react mainly at SH1, gave very similar results, as shown in Figs. 5–7. Again, *M* is constant as $1 - Me$ varies from 0 to close to 1. A 240-min treatment with 20 μM FDNB gave 80% labeling at SH1 and very little label at SH2. It is interesting that for FDNB, the value of *N* was only 7.1, as compared to 16 for IASL.

NPM

The behavior of NPM is slightly more complicated than that of IASL and FDNB. NPM modification causes the EDTA ATPase activity (*Me*) to decrease monotonically; the calcium ATPase activity (*Mc*), however, initially increases, but then decreases with long exposures (Fig. 8). The relationship between *M* and $1 - Me$ is shown in Fig. 9. For $1 - Me < 0.8$, *M* is more or less constant. This implies relatively specific labeling at SH1. However, for $1 - Me > 0.8$, *M* decreases. As derived in the equations, and as seen from the increase in *Z* shown in Fig. 10, this implies labeling of SH2 groups. Nonetheless, Fig. 10 shows that about 80% of the SH1 groups can be specifically modified with NPM before a significant number of SH2 groups react with NPM. With 100 μM NPM a peak of 78 $\pm$ 5.3% occurs at 60 min, and with 150 μM NPM, a peak value of 84 $\pm$ 6.6% is attained at ~30 min. As might be expected, with 150 μM NPM, the rise and fall below 80% occurs more rapidly on both sides of the peak. For $1 - Me < 0.8$, the average value of *M* is 4.5 $\pm$ 0.16, implying that *N* is ~5.5.

An important control for the calcium ATPase measurement

In measuring the calcium ATPase activity, it is important to ascertain that the amount of myosin in the assay mixture is such that the P_i hydrolyzed is proportional to the amount of myosin. We never found this to be a problem in control fibers, or in fibers treated in a way that decreases the calcium ATPase activity, but in fibers treated in a way that increases the calcium ATPase activity it was found necessary to check this. The potential measurement artifact is

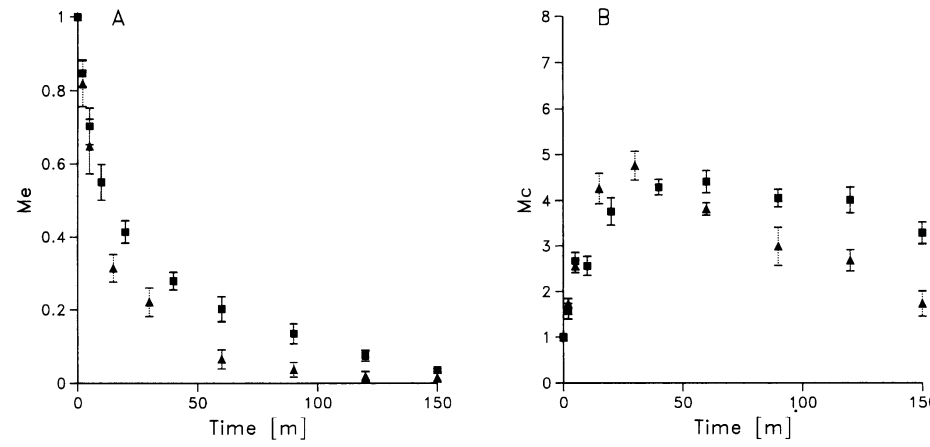

FIGURE 8 Normalized EDTA and calcium ATPase activities (*Me* and *Mc*) as a function of treatment duration when small muscle bundles are treated with NPM at 5°C. ■, Data obtained with 100 mM NPM. ▲, Data obtained with 150 mM NPM.

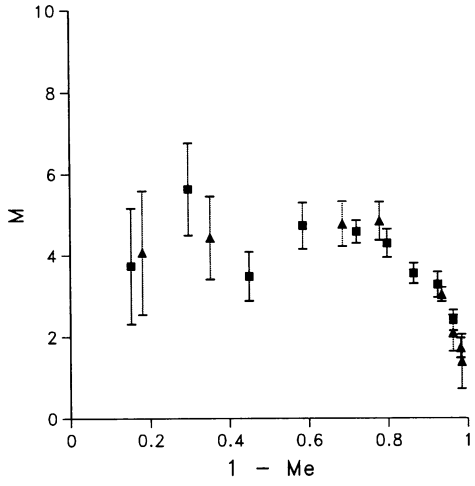

FIGURE 9 The *M* versus 1 − *Me* relationship for NPM. □, Data obtained with 100 mM NPM. △, Data with 150 mM NPM. Because of the large number of points, the horizontal error bars have been omitted, but are the same order of magnitude as seen in the other two *M* versus 1 − *Me* plots.

illustrated in Fig. 11, which gives results from a fiber bundle treated with 0.5 mM IASL for 2 h. For each of the protein amounts used in the assay (given in micrograms on the side of Fig. 11 *A*), the OD (amount of P_i hydrolyzed) is linear as a function of time, but the amount of P_i hydrolyzed per unit time is proportional to the amount of myosin (as it should be), only for the lower protein amounts. In this paper, all measurements were done with protein amounts in this linear range. Although this is potentially a serious problem, so far as we are aware, previous studies also have been in this linear range. The important point from Fig. 11 is that anyone using the technique of Lanzetta et al. to measure myosin ATPases should either report having done this control, or, at the very least, report the concentration of myosin used in the assay.

DISCUSSION

We have been able to summarize the conclusions from many experiments involving the alkylation of myosin's two most reactive sulfhydryls, SH1 and SH2, in terms of a few simple assertions. From these assertions, we have been able, in a few simple equations (Eqs. 1–4), to relate the high-salt EDTA and calcium ATPase activity of myosin to the fractional alkylation of SH1 and SH2 as the alkylation duration is varied. The theoretical formulation predicts a simple graphical means of displaying the data that makes clear the specificity of a given alkylating agent. We tested our new analysis paradigm on two compounds that have been verified as specific modifiers of SH1, IASL and FDNB (Seidel et al., 1970; Reisler et al., 1977). Graphical analysis (Figs. 3 and 6) confirms that both compounds fit the profile predicted for labels specific for SH1. This lends support not only to those prior studies, but also to the correctness of our mathematical and graphical approach. Accordingly, we used our approach to determine the treatment protocol that maximizes the fraction of NPM label specifically on SH1.

The equations derived here build upon the formulation presented previously by Crowder and Cooke (1984). The similarity of the two approaches can be seen by comparing their analysis of their figure 6 with our analysis of Fig. 3, both curves showing data on the high-salt EDTA and calcium ATPases after treatment with 0.5 mM IASL at ~0°C. They plotted the calcium ATPase activity on the ordinate versus the fractional EDTA ATPase (*Me*) on the abscissa. We have plotted (*Mc* − 1)/(1 − *Me*) versus 1 − *Me*. Clearly the abscissas are identical, especially because their axis runs from 1 to 0 and ours from 0 to 1. Whereas in our figure labeling of SH2 with increased labeling times is manifested as a downward deviation from a flat (horizontal) line, in their figure labeling of SH2 is manifested as a downward deviation from a straight line. Whereas in our formulation the *y* intercept as 1 − *Me* approaches zero gives the multiplicative increase (as *N* − 1) in the calcium ATPase activity for SH1 labeling, in their formulation it is the slope that is equal to *N* − 1 (or would be, if the ordinate were relative calcium ATPase activity in their figure 6).

In comparing the formulation of Crowder and Cooke with that presented here, the important thing to make clear is that the current authors are heavily indebted to Crowder and Cooke for their ideas, many of which are incorporated into

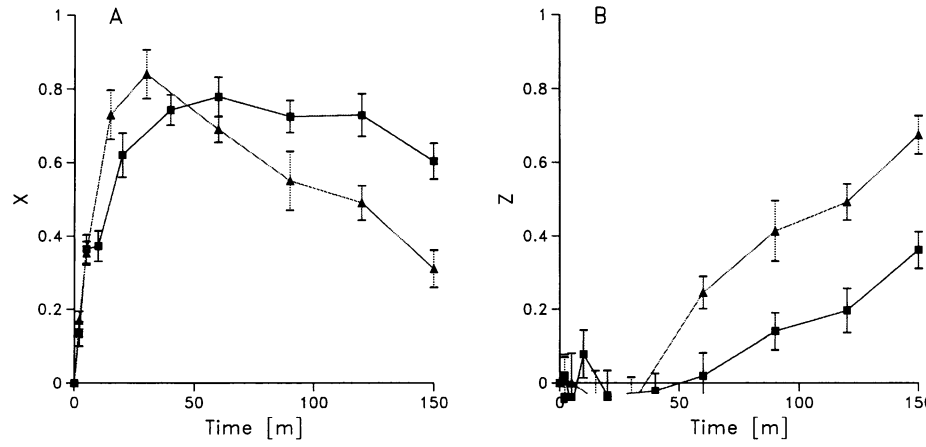

FIGURE 10 (*A*) Fractional labeling of SH1 by NPM (*X*). (*B*) Fraction of myosin heads with SH1 and SH2 labeled (*Z*). ■, 100 mM. ▲, 150 mM.

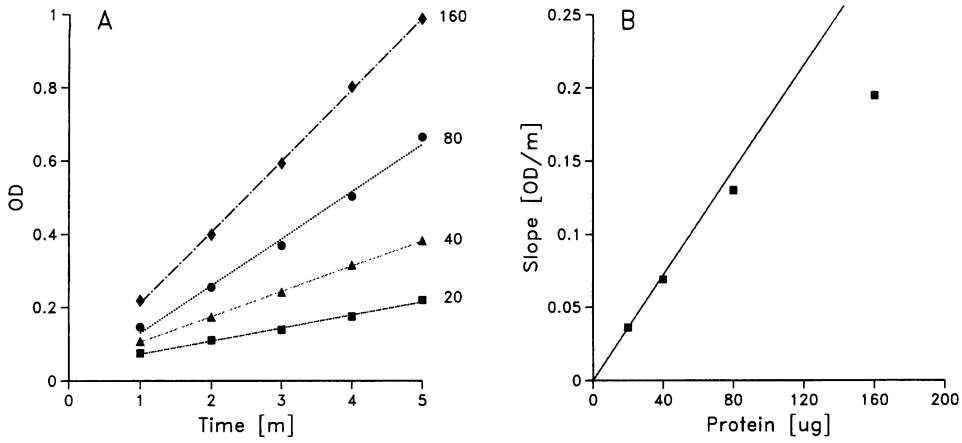

FIGURE 11 Optical density changes recorded during measurement of the calcium ATPase activity of a small bundle of rabbit psoas fibers treated with 0.5 mM IASL for 2 h. (*A*) O.D. as a function of time for 20, 40, 80, or 160 μg of protein in the incubation tube. Note the linear increase in O.D. for each protein amount. However, also note (*B*) that the rate of O.D. increase, which should be proportional to the rate of P_i hydrolysis, is not proportional to protein amount (——) for the higher amounts. For untreated fiber bundles, the rate of optical density change was always proportional to the amount of protein in the incubation tube over this range.

our analysis. We believe, furthermore, that our formulation of the problem represents a significant enhancement of their earlier formulation. This is especially true for the case in which there is significant SH2 labeling. We believe that our approach is an improvement upon that of Crowder and Cooke for the case of significant SH2 alkylation for three reasons. First, we believe our formulation is simpler. Second, we know of no simple expression for the fractional alkylation of SH2 using the Crowder and Cooke formulation. Third, although presumably one could apply our equations to the formulation of Crowder and Cooke to derive such an expression, with real-life data their formulation would be more difficult to use than ours. This is because in both formulations, the fraction of SH2 sites labeled ends up being derived from the deviation of the y variable from a straight line. In our case, deviation from a horizontal line, we need only be concerned about vertical uncertainties in M. Because M is a normally distributed measured variable, there are simple, well-known techniques for doing this. In the Crowder and Cooke formulation, in which one is looking at deviations from a sloped line, it is unclear whether to use the horizontal, vertical, or perpendicular deviation of Mc, and it is unclear how to take experimental uncertainties in the horizontal variable, Me, into account.

Having discussed carefully the differences in the formulations used by ourselves and Crowder and Cooke, we should also point out the slight difference between the data of Crowder and Cooke's figure 6 and our Fig. 3. The deviation from a straight line seen in their data with longer labeling times is more than the deviation from a flat line seen in our data. Our data suggests more specific labeling of SH1. It is impossible to rule out the possibility that the specificity of the IASL we were able to purchase in 1996 was better than that obtainable in 1984. Although our treatment buffer was slightly different from that of Crowder and Cooke, this is not the explanation for the small difference in data, because we obtained an identical result using their buffer (unpublished observations). Although, conceivably, the data of Crowder and Cooke may have been subjected to the artifact shown in Fig. 11, we have no evidence that this is the case.

Maleimide spin label has been reported to be less specific for SH1 than either IASL or FDNB (Reisler et al., 1977). Comparison of Figs. 3, 6, and 9 reveals that *N*-phenylmaleimide also is less specific. Nonetheless, Fig. 10 shows that by limiting labeling time to <60 min with 100 μM NPM, or <40 min with 150 μM, it is possible to avoid significant SH2 labeling. The maximum SH1 labeling attained is ~80%. This amount of label specifically on SH1 should prove sufficient to compare and contrast the physiological effects of SH1 versus SH2 labeling.

In summary, we have used several assumptions suggested by Crowder and Cooke (1984) to relate a muscle fiber's high-salt EDTA and calcium ATPases with the fractional alkylation of SH1 and SH2 sites. We developed a graphical display that provides a clear visualization of the selectivity of the alkylating agent for SH1 over SH2. A few simple equations for quantitating the fractional alkylation of SH1 and SH2 sites were presented, and the results were used to examine the alkylation of SH1 and SH2 by NPM.

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