Phenomenological Analysis of the Kinetics of the Production of Interchain Disulfide Cross-Links in Two-Chain, Coiled-Coil Proteins by Reaction with 5,5′-Dithiobis(2-nitrobenzoate)†

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ABSTRACT: In parallel, registered, two-chain coiled-coil proteins such as tropomyosin, paramyosin, or myosin rods, a cysteine may appear at the same level on each chain. These may be cross-linked by reaction with 5,5′-dithiobis(2-nitrobenzoate) (NbS_2). This technique is useful in several types of studies of such proteins. It is generally accepted that the NbS_2, ordinarily at high molar concentration relative to protein, first reacts in a pseudo-first-order process with one cysteine, blocking it by formation of a mixed disulfide (NbS–S–protein); then, the second cysteine either reacts similarly, producing a doubly blocked site, or attacks the mixed disulfide in a sulfhydryl–disulfide interchange to produce an interchain cross-link. Here, the coupled differential equations for such a system are first set up and solved for a molecule with one such cross-linkable site on the assumption that the condition of one sulfhydryl (blocked or unblocked) does not alter the kinetics of its neighbor. Solutions are presented, giving the concentrations of all species as a function of time and of the rate constants for blocking and for cross-linking. It is also shown how nonreducing sodium dodecyl sulfate/polyacrylamide gel electrophoresis experiments on the products allow a determination of the ratio of the two rate constants. The theory is applied to illustrative data on tropomyosin and shown to fit well. Values of both rate constants emerge. A similar analysis is made for the case where two independent cross-linkable sites exist per molecule. The equations are applied to extant data on short subunit 2 of myosin rod. The fit is less satisfactory, but it is not yet clear whether this is caused by deficiencies of the kinetic model or uncertainties in the data. Finally, solutions are presented for the case where the kinetics of one sulfhydryl at a site does depend on the state of the other sulfhydryl.

In the native state, the molecules of tropomyosin, myosin rod, and paramyosin are α-helical coiled coils in which two α-helical chains are arranged in parallel and in register and subjected to a slight supertwist (Fraser & MacRae, 1973). The roles played by these proteins in the functioning muscle span a wide range, being structural, regulatory, and contractile. This pairing of α-helices can result in matching of a cysteine at a given position with another on the neighboring chain. Consequently, disulfide cross-links can be formed; indeed, the procedure has become an important tool in proving the parallelism and registration of the two chains (Johnson & Smillie, 1975; Lehrer, 1975; Stewart, 1975), in probing the structural integrity near the cysteine sites (Lehrer, 1978; Lehrer & Joseph, 1985), in assaying a population of coiled coils for "hybrid" species, i.e. coiled coils in which the two α-helical members do not have the same sequence (Holtzer et al., 1984), and in studying the helix-to-random-coil transition (Lehrer, 1978; Holtzer et al., 1983).

Of the various methods, cross-linking by 5,5′-dithiobis(2-nitrobenzoate) (NbS_2)[1] has perhaps been the most used (Lehrer, 1975, 1978). This reaction has a dual nature (Lehrer, 1975). First, the NbS_2 reacts with a free sulfhydryl with formation of a mixed disulfide (NbS–S–protein) and liberation of an NBS^- anion ("blocking"). The blockage can be readily reversed with DTT. The second step may be reaction of another NbS_2 with the sulfhydryl on the second chain, forming a doubly blocked site and liberating a second NbS^-. Instead, however, the free sulfhydryl on the second chain may attack the mixed disulfide and force a sulfhydryl–disulfide interchange, resulting in a disulfide cross-link and liberation of another NbS^- anion ("cross-linking").

Two limiting cases are immediately discernible. If the rate of blocking is very great compared with that of cross-linking, then all sulfhydryls will be blocked before any cross-linking can occur. On the other hand, if vice versa, the end result is all molecules cross-linked and no blocked sulfhydryls. Comparable rates yield something in between.

The nature of the actual reaction in a given case may be probed by several types of experiments (Lehrer, 1975). Since each reaction, whether blocking or cross-linking, frees an NbS^- anion (a strong absorber at 412 nm), the overall process may be studied by following this absorbance vs. time, but this experiment merely measures the fraction of sulfhydryls reacted without distinguishing how. Two further experiments, both relatively straightforward, can supplement data on the time course of absorbance (Lehrer, 1975). Both probe the system after a long time. In the first, the reacted protein is dialyzed and run on nonreducing $NaDodSO_4$/PAGE, and the final fraction of cross-linked material (dimer band) is determined by scanning. In the second experiment, the reacted protein is dialyzed and then reacted in $NaDodSO_4$ with excess DTT,

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[1] In the text, we use NbS_2 [which is understood to stand for $(NbS)_2$] to indicate 5,5′-dithiobis(2-nitrobenzoate). In the mathematical treatment, however, even this is too cumbersome and we simply employ the symbol x_2 for the same compound. Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $NaDodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; SS2, short subfragment 2 of myosin rod.

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quantitatively liberating an NbS^- from every blocked sulfhydryl and thereby revealing the final fraction of total sulfhydryls that were blocked.

This qualitative picture of the reaction is generally accepted, and it has long been apparent that the reaction kinetics dictate the end results (Lehrer, 1975; Lu & Lehrer, 1984). However, the use of the cross-linking technique may be enhanced by a formal kinetic analysis. In spite of increasing interest in use of this probe, such an analysis does not seem to have been carried out even on the phenomenological level. We present such an analysis here, in which the relevant coupled kinetic differential equations are solved to obtain the concentration of each molecular species as a function of time and of the rate constants. The equations derived can be used to test the correctness of the basic picture and to interpret the three types of experiments described above. As we will see, the result of either the PAGE or the DTT experiment at a long time, even in the absence of any kinetic studies, places an important constraint on the relative rate constants of the blocking and cross-linking reactions.

Experimental Procedures

The experiments were performed in collaboration with Dr. Marilyn Emerson Holtzer. Preparation of $\alpha\alpha$-tropomyosin from cardiac muscle and its reduction with DTT were as described previously (Holtzer et al., 1983). Dialysis under N_2 was vs. 0.5 M NaCl–0.05 M NaP_i, pH 7.4, which was the solvent for the experiments. Reaction at 20 °C was initiated by rapidly adding 20 μL of 100 mM NbS_2 to 2.00 mL of 1.16 mg/mL protein solution in a 1-cm absorbance cell. The solution was mixed by rapid hand-inversion. The clock was started when the sample was placed in the spectrophotometer. The absorbance at 412 nm of the solution (vs. a blank containing the same concentration of NbS_2) was measured at 20-s intervals until A_{412} remained constant. The precise zero of time was established by plotting log $[(A_\infty - A)/A_\infty]$ vs. clock time to determine the small, uniform shift needed to assure that the data extrapolated to zero ordinate at zero time. All clock times were then corrected by that constant amount. Absorbances were determined by using a Shimadzu UV-160 UV–vis recording spectrophotometer in the time-scan mode.

Theory

Coiled Coils with a Single Cross-Linkable Site. The prototypical example here would be $\alpha\alpha$-tropomyosin, wherein each 284-residue chain has only one cysteine, C-190. We use x_2 to stand for NbS_2 species and x for the NbS^- anion or radical. Each helical chain is designated α; hence, $\alpha\alpha$ is the intact, non-cross-linked (reduced) coiled coil. We designate the cross-linked molecule α_2, so the present notation differs from conventional chemical parlance in that here we make a distinction between $\alpha\alpha$ and α_2. However, we make no distinction between singly blocked species $\alpha\alpha$x and x$\alpha\alpha$.

With these definitions, the three relevant chemical reactions can be written:

$$\alpha\alpha + x_2 \xrightarrow{2k_1} \alpha\alpha x + x \quad (1)$$

$$\alpha\alpha x + x_2 \xrightarrow{k_1} x\alpha\alpha x + x \quad (2)$$

$$\alpha\alpha x \xrightarrow{k_2} \alpha_2 + x \quad (3)$$

In eq 1–3, all back-reactions are assumed negligible. The appropriate rate constant is indicated above each reaction arrow. In the case of reactions 1 and 2 these are pseudo-first-order constants; this is appropriate, since the molar concentration of NbS_2 is normally much greater than that of the protein (Lu & Lehrer, 1984). The rate constant k_1 is that inherent to blocking at a single site, as in reaction 2. The rate constant for an unblocked molecule is simply assumed to be double that; i.e., we assume that blocking a sulfhydryl on one chain does not affect the rate constant for blocking the sulfhydryl on the adjacent chain. The factor of 2 then accounts completely for the second site. A discussion will be given on what happens when this independence assumption is relaxed.

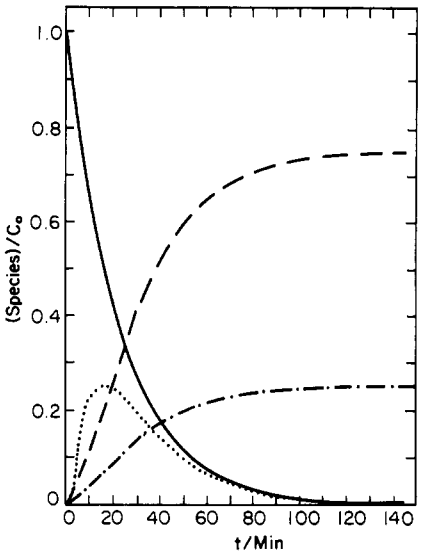

FIGURE 1: Illustrative theoretical curves for relative concentrations of various protein species. All curves for one cross-linkable site with $k_1 = 0.022$ min^{-1} and $k_2 = 0.066$ min^{-1}. Solid curve, unreacted species; dotted curve, singly blocked species; dot–dashed curve, doubly blocked species; dashed curve, cross-linked species. Asymptote for doubly blocked species is $k_1/(k_1 + k_2) = 0.25$; asymptote for cross-linked species is $k_2/(k_1 + k_2) = 0.75$.

There are four possible protein species: unreacted, singly blocked, doubly blocked, and cross-linked. The concentration of each is to be evaluated as a function of time. The small-molecular species x is also of interest, since the time course of its concentration is experimentally accessible.

The rate equations corresponding to the reaction scheme can be written down immediately, and this set of differential equations is readily solved for each of the species concentrations. The reader is referred to the supplementary material for the mathematical details (see paragraph at end of paper regarding supplementary material). The results, giving the molar concentration of each species, are conveniently expressed in terms of two derived constants, $k_+ = k_1 + k_2$ and $k_- = k_2 - k_1$, and of C_0, the formal concentration of protein (as two-chain species):

$$(\alpha\alpha) = C_0 e^{-2k_1 t} \quad (4)$$

$$(\alpha\alpha x) = 2C_0(k_1/k_-)(e^{-2k_1 t} - e^{-k_+ t}) \quad (5)$$

$$(x\alpha\alpha x) = C_0[k_1/(k_+k_-)](k_- - k_+e^{-2k_1 t} + 2k_1 e^{-k_+ t}) \quad (6)$$

$$(\alpha_2) = C_0[k_2/(k_+k_-)](k_- - k_+e^{-2k_1 t} + 2k_1 e^{-k_+ t}) \quad (7)$$

$$(x) = C_0[2/k_-][k_- - k_2 e^{-2k_1 t} + k_1 e^{-k_+ t}) \quad (8a)$$

The time dependence of the concentrations of the various protein species is shown in Figure 1 for representative values of k_1 and k_2.

The absorbance at 412 nm, being directly proportional to (x), is also obtained from eq 8a. Kinetic data are usually expressed in terms of the fraction of sulfhydryls remaining intact (f_i), which algebraic condensation shows to be given by

$$f_I = [(x)_\infty - (x)]/(x)_\infty = (A_\infty - A)/A_\infty = (k_2/k_-)e^{-2k_1 t} - (k_1/k_-)e^{-k_+ t} = f_2 e^{-2k_1 t} - f_1 e^{-k_+ t} \quad (9a)$$

Thus, as eq 9a shows, the usual experimental variable should be fit to a difference of two positive exponentials whose coefficients are related by $f_2 - f_1 = 1$. In the event that k_1 and k_2 are comparable in magnitude, both may be obtained from a computer fit of the data. If $k_1 \gg k_2$, then a single exponential ($e^{-k_1 t}$) would fit the data. However, if $k_2 \gg k_1$, then a single exponential ($e^{-2k_1 t}$) would also fit the data. Additional experiments are needed, therefore, that bear on the relative values of k_1 and k_2 if interpretable rate constants are to be determined.

As noted, experiments at long time can give the fraction cross-linked and/or the fraction of sulfhydryls blocked. Equation 7 gives for the fraction dimer (g_d) at infinite time

$$g_d = (\alpha_2)_\infty / C_0 = k_2/k_+ = k_2/(k_1 + k_2) \quad (10a)$$

Thus, since integration of the gel scan provides g_d, we can immediately obtain from this $g_d/(1 - g_d) = k_2/k_1$, the ratio of the two rate constants. If the model is correct, this kinetic information is thus available from this single experiment, performed after the reaction is over, even when no information on the time course is available. Of course, if through some side reaction not considered in our scheme some molecules are unable to cross-link, then g_d will be too small and will merely provide a greatest lower bound for k_2/k_1.

As is immediately evident (and is also seen from the above equations), the only protein species allowed by the model at a very long time are cross-linked molecules and doubly blocked molecules. From eq 6 we can obtain the fraction of sulfhydryls blocked after long times (f_B), which is determinable from subsequent reaction with DTT and is, of course, the same as the fraction of non-cross-linked molecules ($g_m = 1 - g_d$):

$$f_B = g_m = 2(x\alpha\alpha x)_\infty/(2C_0) = k_1/k_+ = k_1/(k_1 + k_2) \quad (11)$$

This experiment is related to the PAGE experiment, as is well recognized, and gives no new information, but is a useful check (Lehrer, 1975). Thus, the PAGE or DTT experiment enables one to determine the relative values of k_1 and k_2. Use of this experimentally determined value of $r \equiv k_2/k_1$ in eq 9 then reduces to one the number of parameters requiring evaluation from a fit to the kinetic data.

Coiled Coils with Two Cross-Linkable Sites. Prototypical cases of this sort are $\beta\beta$-tropomyosin, wherein each β chain has a cysteine at positions 36 and 190, and "short subfragment 2" (SS2) of myosin rod which has them at positions 66 and 108. In each case, the two cross-linkable sites are distant from one another, and we will assume that the two sites are independent, but not equivalent.

We refer to the sites as site a and site b, respectively. Since they are assumed to be independent, it is evident that the equations derived in the previous section still hold *at each independent site.* We have only to put them together properly so as to describe the two-site case. Since the rate constants for blocking and cross-linking may each be different at the different sites, we define k_{1a} (k_{1b}) and k_{2a} (k_{2b}) for reactions like eq 1–3 occurring at site a (b). We also define, analogously

$$k_{+a} = k_{1a} + k_{2a} \qquad k_{+b} = k_{1b} + k_{2b}$$
$$k_{-a} = k_{2a} - k_{1a} \qquad k_{-b} = k_{2b} - k_{1b} \quad (12)$$

In the following, we focus narrowly on the readily determined experimental quantities: (1) the concentration of NbS^- liberated by the reaction as a function of time, as usually expressed as $f_I = (A_\infty - A)/A_\infty$, i.e., the fraction of sulfhydryls remaining unreacted; (2) the fraction of cross-linked dimer (g_d) appearing on $NaDodSO_4$/PAGE after the reaction is over; (3) the amount of NbS^- liberated from the protein by DTT treatment after the reaction is over, again expressed as f_B, the fraction of sulfhydryl sites blocked.

The NbS^- liberated in the reaction with NbS_2 is the simple sum of that liberated at each site. The latter is given by an equation like (8). We thus have for the two-site case

$$(x) = C_0[(x)_a + (x)_b] = 2C_0[2 - (k_{2a}/k_{-a})e^{-2k_{1a}t} + (k_{1a}/k_{-a})e^{-k_{+a}t} - (k_{2b}/k_{-b})e^{-2k_{1b}} + (k_{1b}/k_{-b})e^{-k_{+b}t}] \quad (13)$$

where $(x)_a$ [$(x)_b$] is the concentration of NbS^- due to reaction at site a (b) alone. Such quantities are not to be construed as mere convenient fictions. It is possible under some circumstances to block a given site permanently and selectively (e.g., with iodoacetamide). Addition of NbS_2 to such a modified protein would then yield the time course of $(x)_a$ separately. Repeating the experiment on the original unblocked protein would provide (x); hence, subtraction would give $(x)_b$ as a function of time, i.e., the time course at site b.

Recognizing that $(x)_\infty = 4C_0$ and using eq 13 yield immediately for the fraction of sulfhydryls remaining intact:

$$f_I = (f_{2a}/2)e^{-2k_{1a}t} - (f_{1a}/2)e^{-k_{+a}t} + (f_{2b}/2)e^{-2k_{1b}t} - (f_{1b}/2)e^{-k_{+b}t} \quad (14)$$

where $f_{2a} = k_{2a}/k_{-a}$ etc. and $f_{2a} - f_{1a} = f_{2b} - f_{1b} = 1$. As (14) shows

$$f_I = (f_{Ia} + f_{Ib})/2 \quad (15)$$

that is, the fraction of sulfhydryls remaining intact at any time is the arithmetic average of what it would be if each site were alone active.

We turn next to the population of cross-linked chains as would be revealed by $NaDodSO_4$/PAGE at infinite time. Since the sites are independent, the results of the previous section describe the situation at each site. For example, it is evident that the fraction of molecules cross-linked at site a is k_{2a}/k_{+a} etc. This site description then leads straightforwardly to expressions for the relative populations of each of the three possible types of cross-linked molecules (cross-linked at site a, cross-linked at site b, and cross-linked at both sites). The details can be found in the supplementary material. If the $NaDodSO_4$/PAGE system used is appropriate, the dimer region may show three bands with the appropriate relative populations. In general, the resolution is not expected to be that good, but it is always possible to resolve dimer region from monomer. The total fraction of cross-linked material is given by summing the three species populations and, as shown in the supplementary material, is given by

$$g_d = (g_d)_a + (g_d)_b - (g_d)_a(g_d)_b \quad (16)$$

Again, in (16) we use $(g_d)_a$ [$(g_d)_b$] to signify the final dimer fraction that would be obtained if only site a (b) were cross-linkable. In this experiment, then, the observed value is not the simple arithmetic average of the two site values, but a more complicated function of them.

Finally, we inquire about the release of NbS^- by DTT from doubly blocked sites at long time. As in the gel experiment, there are three relevant molecular species (doubly blocked at site a, doubly blocked at site b, and doubly blocked at both sites) whose populations can be given in terms of the site-description parameters (see supplementary material). When these are summed, we obtain for the final fraction of sulfhydryls blocked:

$$f_B = [(f_B)_a + (f_B)_b]/2 \quad (17)$$

again giving a result that is the arithmetic mean of the result that would be gotten if each site were the sole cross-linkable position.

There is an important difference in the two-site and one-site cases. In the one-site case, the PAGE and DTT experiments on the final state are redundant, i.e., $g_d = 1 - g_m = 1 - f_B$. Thus, the only kinetic information provided is the ratio k_2/k_1, and it can be obtained from one or the other. This is not true in the two-site case, where both results are needed to obtain analogous information for *each* of the sites. This can be done by solving eq 16 and 17 simultaneously. The result is a quadratic whose solution is

$$(g_d)_a = 1 - (f_B)_a = 1 - f_B \pm [f_B^2 - 1 + g_d]^{1/2} \quad (18)$$

Reinsertion of this into (17) gives

$$(g_d)_b = 1 - (f_B)_b = 1 - f_B \mp [f_B^2 - 1 + g_d]^{1/2} \quad (19)$$

Equations 18 and 19 allow calculation from g_d and f_B of two values, one of which gives $k_{2a}/k_{1a} = (g_d)_a/[1 - (g_d)_a]$ and one $k_{2b}/k_{1b} = (g_d)_b/[1 - (g_d)_b]$, i.e., the ratio of blocking to cross-linking rate constants for each of the two sites, but it is impossible to tell which ratio applies to which site without information from additional experiments. If such experiments are available, then both ratios are known, and this allows kinetic data to be fit to eq 14 with only two adjustable parameters. Note also that eq 18 and 19 require that $f_B^2 \geq (1 - g_d)$, a relation that may be useful in testing the self-consistency of experiments in which both are measured.

Application

Tropomyosin. The earlier gel and DTT experiments of Lehrer on rabbit skeletal tropomyosin show remarkable self-consistency (Lehrer, 1975). Examination of Figure 3 of the work cited indicates a final value of 93% cross-linked dimers from PAGE. For the same sample, the reported liberation of 0.53 mol of NbS^-/mol of protein by DTT (Lehrer, 1975), when corrected for C-36 content of the β chains, implies that 95% of the molecules must have been cross-linked at C-190, in excellent agreement with the gel experiments.

What has not been noted before is that a cross-linked dimer content of somewhat above 90% implies that, in this system, $k_2/k_1 \cong 10$. This result could be checked by further experiments in which k_1 (which is a pseudo-first-order rate constant) is varied by reducing (or raising) somewhat the concentration of NbS_2, whereupon the yield of cross-linked dimer ought to be raised (or reduced) accordingly.

To our knowledge, results of only one study of the kinetics have been reported on tropomyosin, but no actual data points are given (Lu & Lehrer, 1984). Some data were therefore generated here on $\alpha\alpha$-tropomyosin at 20 °C which will serve to illustrate the interpretation (M. E. Holtzer and A. Holtzer, unpublished experiments). For $\alpha\alpha$-tropomyosin, the experiment gives $g_d \cong 90$–95% (Holtzer et al., 1983), just as in the skeletal protein.

Our illustrative data are plotted on Figure 2 along with theoretical curves using $k_1 = 0.12$ min^{-1} $= k_2/10$. It is apparent, considering the normal experimental scatter, that the theory fits the data well. The k_2/k_1 ratio is a bit high to allow a precise determination of k_2. It is certainly fair to say, however, that $k_2 \geq 1.2$ min^{-1}, so a greatest lower bound has been placed upon it. These values of k_1 and k_2 were obtained from the best computer fits to the functional form of eq 9. For each fit, a ratio k_2/k_1 was assumed and the best k_1 evaluated. Then k_2/k_1 was varied and the process repeated. The best fit of all was obtained for a ratio of 10, which is consistent with the PAGE and DTT experiments. In our experiments the

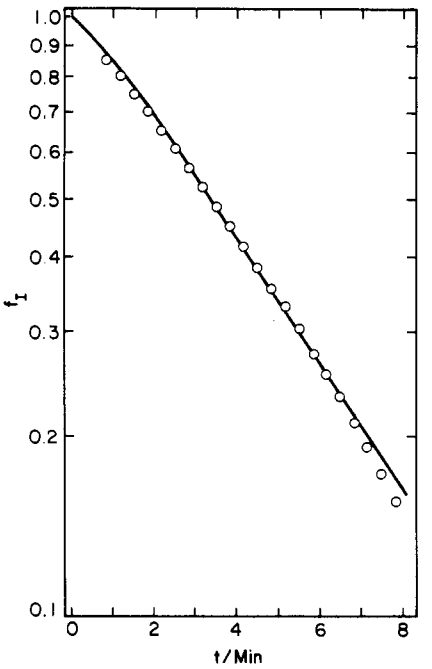

FIGURE 2: Semilog plot of fraction of intact sulfhydryl in $\alpha\alpha$-tropomyosin vs. time after addition of NbS_2. Circles are data of M. E. Holtzer and A. Holtzer (unpublished experiments) at pH 7.4 and 20 °C, protein concentration 1.15 mg·mL^{-1} (0.036 mM), and NbS_2 concentration 0.99 mM in 0.5 M NaCl–0.05 M NaP_i. Solid curve is calculated from eq 11 with $k_1 = 0.12$ min^{-1} and $k_2 = 1.2$ min^{-1}.

NbS_2 concentration was ~1 mM so the bimolecular rate constant for blocking the protein at 20 °C is 120 L·mol^{-1}·min^{-1}. The corresponding pseudo-first-order rate constant for β-mercaptoethanol at 25 °C is ~72 min^{-1}, many times k_1 or k_2 for tropomyosin even allowing for the difference in temperature (Lu & Lehrer, 1984).

Short Subunit 2 of Myosin Rod. The kinetic data on this substance show a fast phase and a slow phase (Lu & Lehrer, 1984). Extrakinetic experiments show that blocking, but no cross-linking, occurs in the fast phase and that in the final product no cross-links are present at C-66 while only about 20–40% of the molecules are cross-linked at C-108 (Lu & Lehrer, 1984). It is evident, then, that blocking is much more rapid at C-66 than it is in tropomyosin and that cross-linking is considerably *slower* than blocking at that position. Since it is likely that 10% cross-linking at C-66 would have been seen, the results of the Theory section then indicate that $k_{2a}/k_{1a} < 0.1$, designating C-66 as position "a". Indeed, it may be that $k_{2a}/k_{1a} \ll 0.1$. The finding for C-108 show somewhat more definitely that $k_{2b}/k_{1b} \cong 0.25$–0.67. Thus, the PAGE experiments place constraints that reduce the number of parameters required to fit the data to two.

Figure 3 shows our attempt to fit the extant data on SS2 to eq 14. Data points were obtained from an enlargement of Figure 1 of Lu and Lehrer (1984). The theoretical curve is for $k_{1a} = 0.91$ and $k_{2a} = 0.091$ ($r_a = 0.1$) and for $k_{1b} = 0.062$ and $k_{2b} = 0.016$ ($r_b = 0.25$). This fit is the best that can be obtained within the rather wide limits set by the PAGE experiments. Unfortunately, the fit shown, although best, is not very good. Although experimental variance may be substantial, the deviation seems systematic. At present, however, it is impossible to tell whether the deviations are caused by a failure of our kinetic model to accord with the physical system or by some internal inconsistency in the data. The former would apply if, say, kinetics of either blocking or cross-linking at C-66 depended upon the state of the sulfhydryl at C-108, a contingency that is perhaps not as remote as might

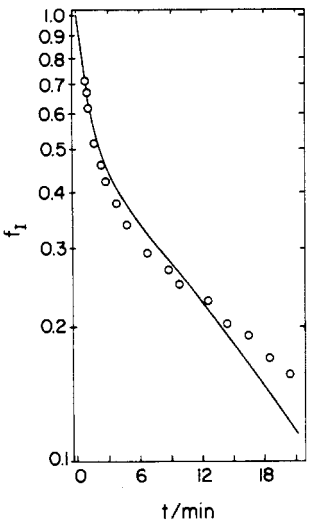

FIGURE 3: Semilog plot of fraction of intact sulfhydryl in short subfragment 2 of myosin rod vs. time after addition of NbS_2. Circles are data from Figure 1 of Lu and Lehrer (1984) at pH 7.5 and 16 °C, protein concentration ~0.25 mg·mL^{-1} (~0.006 mM), and NbS_2 concentration 1.12 mM in 0.5 M NaCl–1.8 mM Hepes–1 mM EDTA. Solid curve is calculated from eq 16 with k_{1a} = 0.91, k_{2a} = 0.091, k_{1b} = 0.062, and k_{2b} = 0.0155, all in min^{-1}.

at first be supposed (Lu & Lehrer, 1984). However, the latter is perhaps even more likely since the SS2 system is by no means as well characterized as tropomyosin and the data do not display the same degree of self-consistency. Indeed, only 1.7 ± 0.2 of the 2 sulfhydryls per chain are accounted for in the experiments (Lu & Lehrer, 1984). The possibility that the poor fit would be improved by a small shift in the zero of time (see Experimental Procedures) was explored, but no improvement resulted. In any case, the equations developed here provide a rational framework for further tests of this simplest kinetic model.

Extension to More Complex Kinetic Models

We sketch here the results for the single-site case wherein the blocking rate constant for a sulfhydryl whose neighbor on the adjacent chain is blocked differs from that for one whose neighbor is unblocked. Details of the derivations are analogous to those for the previous case and will be left to the reader.

Reaction equations are as in eq 1–3 except that k_1 in eq 2 now becomes $k_1' \neq k_1$. The new rate equations are modified accordingly. The supplementary material provides further details. The analysis still requires solution of a linear, first-order differential equation. In fact, introduction of the definition $k_+' = k_1' + k_2$ puts it in a *form* identical with the previous with k_+' replacing k_+. Defining also $k_-' = k_+' - 2k_1$ allows the results for the various species to be rather compactly expressed. Of particular interest is the equation for (x), the experimentally determined concentration, which becomes

$$(x) = C_0[2/k_-'][k_-' - (k_+' - k_1)e^{-2k_1t} + k_1e^{-k_+'t}] \quad (8b)$$

The latter yields for the fraction of sulfhydryls intact

$$f_I = f_2'e^{-2k_1t} - f_1'e^{-k_+'t} \quad (9b)$$

where $f_2' = (k_+' - k_1)/k_-'$ and $f_1' = k_1/k_-'$ and $f_2' - f_1' = 1$.

The PAGE experimental result is now readily shown to be

$$g_d = (\alpha_2)_\infty/C_0 = k_2/k_+' = k_2/(k_1' + k_2) \quad (10b)$$

so this experiment gives only $g_d/(1 - g_d) = k_2/k_1'$ and provides no information on k_1. The fraction sulfhydryl blocked is, of course, $f_B = 1 - g_d$ as before and provides only a double check on the PAGE experiment, not new information.

Although a solution has been obtained for this more complex kinetic model, the practical consequences of implementation may be severe. Even with reliable PAGE results (i.e., k_2/k_1') in hand, one must now fit eq 9b to the kinetic data with two unknown parameters, say k_1 and k_1'.

Supplementary Material Available

More explicit exposition and solution of the differential equations describing the mechanism embodied in reactions 1–3 for both the one-site and two-site cases and explicit results for the more complex model (4 pages). Ordering information is given on any current masthead page.

Registry No. NbS_2, 69-78-3.

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