not been observed to be modified in the tyrocidines which we have studied, also occurs unmodified in the cyclic decapeptide gramicidin S which actually is a dimer of it. The reoccurrence of this pentapeptide fragment may have some important implication as regards the biosynthesis or the function of these cyclic peptides in the organism.

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Enzymatic Modification of Myosin by Disulfide Exchange*

David Hartshorne† and Manuel Morales‡

ABSTRACT: With various compounds, and under various conditions, there is studied the exchange between simple disulfides and the SH groups of myosin, and between disulfide myosin and various thiols. The first of these exchanges "modifies" myosin, as regards its nucleoside triphosphatase activity, and the second exchange partially "regenerates" the original myosin. Using ^{35}S-labeled dithiopropionate to modify myosin, and "cold" thiopropionate to regenerate it, one finds that when maximal ATPase activity is reached during modification there is a much greater number of reacted SH groups than when a similar maximal ATPase activity is reached on regeneration. This result is interpretable on the assumption that SH reaction and modification are distinct processes, and therefore not always "tightly coupled."

There is evidence (e.g., Gilmour and Gellert, 1961; Rainford *et al.*, 1964) that, as regards rate of reaction with organic mercurials, the SH groups of myosin [8.6 moles SH/10^5 g myosin, by amino acid analysis (Kominz *et al.*, 1954)] fall into various classes (X, very fast; Y, moderately fast; Z, very slow). It is to be expected that the reactions of myosin SH groups with other reagents will show a similar heterogeneity in reaction rate. In this paper we study, in both directions, reaction by disulfide exchange (Barany, 1959; Stracher, 1963, 1964),

$$R_t\text{—S—S—}R_t + \text{myosin—S}^- \rightarrow \text{myosin—S—S—}R_t + R_t\text{—S}^- \quad (1)$$

We also studied the possible reaction of myosin with thiols (Morales *et al.*, 1957),

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$$R_j\text{—}S^- + \text{myosin—}S^- \rightarrow \text{myosin—S—S—}R_j + 2e^- \quad (2)$$

In the terminology of Rainford *et al.* (1964), the reaction (X → X′) of one or more *particular* groups of the X class modifies myosin so that its (Ca^{2+}, 25°, *p*H 7.0) ATPase activity changes from a native or control (α state) value to a value 3–4 times greater (β state), and the further reaction (Y → Y′) of one or more *particular* groups of the Y class further modifies myosin to the γ state, having no activity at all. The reaction (Z → Z′) of groups of the Z class does not affect activity, and the foregoing transformations for ITPase activity are explicable if ITP itself is considered a modifying agent. In this terminology, maximal ATPase activity on treatment with a reagent is reached when there exists an optimal distribution of enzyme among the α, β, and γ species, i.e., the observed activity is an average of the activity, $\bar{V}$ (max), of so many α molecules, so many β molecules, and so on. Corresponding to this highest average activity there is a certain number ($\bar{n}$) of SH groups reacted, but this number too is an average made up of so many X′ groups, so many Y′ groups, and so on. In this paper we also discuss the consequences of experiments in which the average number of SH groups required to obtain maximal ATPase activity is compared with the (much smaller) average number of SH groups reacted following "regeneration" of nearly γ-myosin back to maximal activity, the regeneration being accomplished by the reversal of reaction (1). Finally, we consider how our various results bear on Stracher's (1963, 1964) interesting scheme for labeling a unique SH group at the active center of myosin.

Experimental Details

In addition to reagent grade ordinary salts, the following substances were used: $Na_2H_2ITP \cdot 3\,H_2O$ (Sigma), crystalline $Na_2H_2ATP \cdot 3\,H_2O$ (Sigma), A-grade lysine·HCl (Calbiochem), and ^{35}S-labeled 3-mercaptopropionic acid at an initial specific activity of 1.1 mc/mmole (New England Nuclear). Bis-β-carboxyethyl disulfide was prepared according to A. Stracher (personal communication); the disulfides of β-mercaptoethanol and glutathione were similarly prepared by oxidation at alkaline *p*H. When thiols such as β-mercaptoethanol were used it was established that under conditions of the experiment the SH titer (Klotz and Carver, 1961) was very nearly the theoretical; disulfides were similarly checked for free SH. Myosin was prepared by the Szent-Gyorgyi method, and checked optically (Rice *et al.*, 1963) for myosin B contamination. The SH titer of myosin was measured spectrophotometrically (Boyer, 1954), but the *p*-mercuribenzoate was incubated with myosin for at least 24 hours at 0° (Gilmour and Gellert, 1961). Myosin concentration was measured by the Folin-Ciocalteau method. In calculating "*N*-fold" concentrations of sulfhydryl reagents, i.e., concentrations of reagent *N* times greater than the concentration of myosin SH, the titer of myosin was assumed to be 8.6 moles SH/10^5 g.

Nucleosidetriphosphatase activity was calculated from Fiske-Subbarow orthophosphate concentration measurements in 0.36 M KCl, 0.03 M Tris chloride, 0.008 M $CaCl_2$, 0.0024 M nucleotide, 25°, *p*H 8.00. Radioactivity was measured with a Nuclear-Chicago gas-flow end-window counter, Model D-47.

Modification of myosin with the compounds tested was achieved by incubation in 0.60 M KCl, 0.10 M lysine, 0°, *p*H 9.30. In some instances (e.g., β-mercaptoethanol) it was necessary to replenish modifier periodically; this was accomplished in a dialysis rig (Morales *et al.*, 1957). In regeneration experiments the myosin being modified with bis-β-carboxyethyl disulfide was diluted 10-fold, at the appropriate time, with regeneration solvent: 3-mercaptopropionic acid in 0.60 M KCl, 0.10 M lysine, 0°, *p*H 9.30. Dialysis against this mixture was then continued for at least 48 hours, with frequent changes of external compartment fluid. Modified myosin was separated from excess modifier by similar dialysis, omitting the 3-mercaptopropionate.

Results

It was found that, when periodically replenished, β-mercaptoethanol is an effective modifier at *p*H 9.3 (Figure 1); however, at *p*H 8 the rate of modification falls to 1/3, and at *p*H 7 there is practically no modification. At equal concentrations and *p*H 9.3, 3-mercaptopropionate and glutathione fail to modify. Parenthetically, it was noted that after myosin had been maximally modified (activated) by β-mercaptoethanol it could be further activated by the disulfide of β-mercaptoethanol.

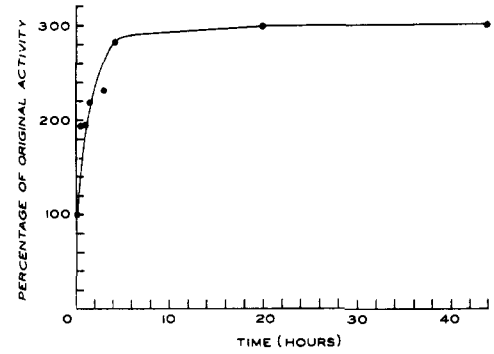

FIGURE 1: ATPase activity of myosin as a function of incubation time in 200-fold molar excess of β-mercaptoethanol.

Modification by disulfides is not as *p*H sensitive as modification by thiols, but it varies widely with reagent structure (Figure 2), and, with 0.49% myosin, modification by dithiopropionate is very sensitive to concentration in the range 20–200 molar excess (Figure 3).

At any time during modification the progress of the reaction can be assessed by freeing an aliquot of protein

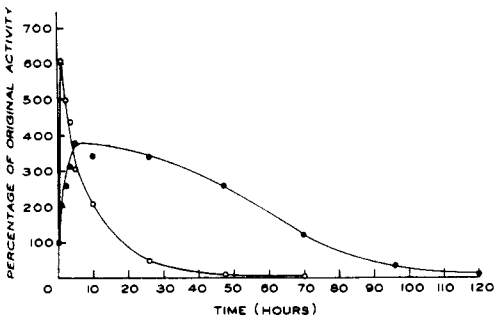

FIGURE 2: ATPase activity of myosin as a function of incubation time in 200-fold molar excess of the disulfide of glutathione (filled circles), and in 50-fold molar excess of the disulfide of β-mercaptoethanol (open circles).

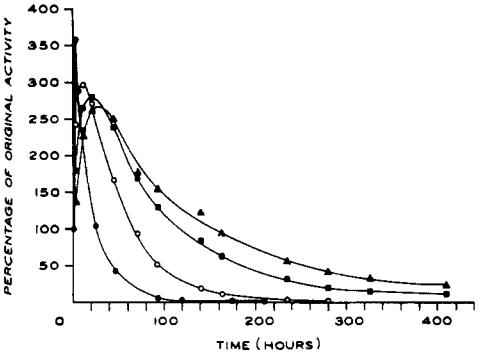

FIGURE 3: ATPase activity of myosin as a function of incubation time in various concentrations of dithiopropionate. Filled circles, 200-fold molar excess; open circles, 100-fold molar excess; filled triangles, 50-fold molar excess; filled squares, 20-fold molar excess.

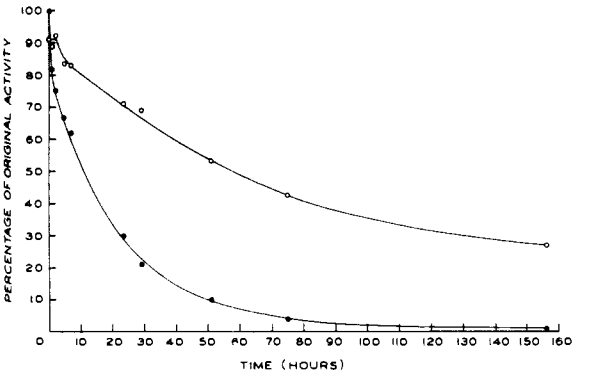

FIGURE 4: ITPase activity of myosin. Filled circles indicate activity as a function of incubation time in 200-fold molar excess of dithiopropionate. Enzyme aliquots whose activity is indicated by filled circles were submitted to 48 hours of regeneration in thiopropionate, and the resulting activities are indicated by open circles.

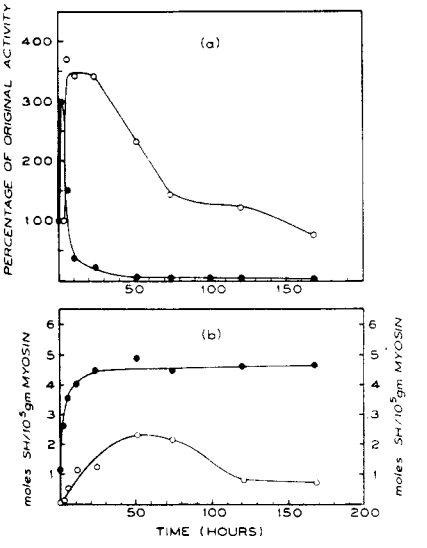

FIGURE 5: Filled circles indicate (a) ATPase activity, and (b) left ordinate, content of reacted SH, as a function of incubation time in 200-fold molar excess of dithiopropionate. When enzyme aliquots with properties indicated by filled circles are submitted to 48-hour regeneration in thiopropionate, the resulting (a) ATPase activity and (b) right ordinate, content of reacted SH, are indicated by open circles.

from modifier, and studying its nucleoside triphosphatase activity under standard conditions. In experiments in which the modifier was ^{35}S-labeled dithiopropionate such measurements are given by the filled circles of Figures 4 (ITPase) and 5a (ATPase). The aliquot can also be characterized by measuring its bound radioactivity and converting this to moles of thiopropionate bound per 10^5 g protein; such measurements are given by the filled circles of Figure 5b. When an aliquot drawn at a certain time is "regenerated," i.e., submitted to 48 hours of dialysis against 3-mercaptopropionate under conditions favorable to the reverse of reaction (1), its ITPase (Figure 4, open circles) and ATPase (Figure 5a, open circles) activities are substantially altered, and its bound radioactivity (therefore the moles of thiopropionate bound per 10^5 g myosin) is substantially reduced (Figure 5b, open circles). Note particularly that the changes induced by short incubation times (up to *ca.* 2.5 hours), though very extensive, are completely reversed by regeneration; this fact has been repeatedly and extensively established in many experiments. To assure that the regeneration achieved in 48 hours was maximal, the kinetics of regeneration was studied in certain aliquots, e.g., those drawn at 24 hours and 121 hours (Figure 6). The effectiveness of regeneration in eliminating labeled thiopropionate residues originally bound during modification can be judged from the horizontal band[1] in

[1] The molecular weight of myosin is controversial, ranging from 4.2 to 6.2 $\times$ 10^5 g, hence the "1 mole SH/mole myosin" line is actually a band.

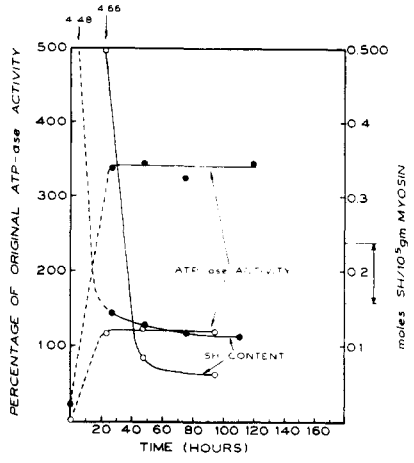

FIGURE 6: Properties of myosin incubated in dithiopropionate for 24 hours (filled circles) and 121 hours (open circles) as a function of time of regeneration in thiopropionate. Left ordinate refers to ATPase activity, and right ordinate to content of reacted SH. Range noted on right ordinate indicates "1 mole SH/mole myosin" level, depending on assumed molecular weight of myosin. The uppermost numbers indicate the moles of initially reacted SH.

Figure 6, which band represents 1 mole of original label per *mole* of myosin. Nonetheless, Figures 5a and 6 show that on certain aliquots significant quantities of labeled thiopropionate remain. Even these quantities are removable when β-mercaptoethanol is used in place of 3-mercaptopropionate (experiments not shown); however, as Figure 1 shows, the former is, or contains, a modifier so it is not a good regenerator if the regeneration of enzyme activity is also to be studied.

Discussion

As already established by Barany (1959) and by Stracher (1963, 1964), disulfides readily exchange with the SH groups of myosin. The present work shows that these groups exhibit a heterogeneity of exchange rate similar to their heterogeneity of mercurial-combining rate, because, as in the latter case, modification with disulfides produces first activation and later inhibition of ATPase. Morales *et al.* (1957) reported that various thiols, when sufficiently ionized and constantly replenished, *activate* myosin very strongly; in the case of the most effective thiol, *S*-β-aminoethylisothiuronium, they noted that the activation turned to inhibition if replenishment stopped. Similar activation by sufficiently ionized thiols has been observed here, and it has been noted that the steady level of activation caused by β-mercaptoethanol (a level considerably less than that achieved by *S*-β-aminoethylisothiuronium) increases further if replenishment is stopped. These "aftereffects" when replenishment is stopped may be due to R_f—S—S—R_f formed adventitiously; if the thiol has already caused maximal activation (*S*-β-aminoethylisothiuronium case) then the aftereffect may diminish the level of activation, while if the thiol has achieved a submaximal activation (β-mercaptoethanol case) then the aftereffect may activate still further. However, it seems very likely that thiol activation goes primarily via reaction (1) because (a) the concentration of disulfide arising in, say, β-mercaptoethanol solutions exposed to the modification conditions would modify at a rate far slower than the rate at which the thiol itself modifies, and (b) elimination of O_2 blocks modification by carefully reduced (treated with sodium borohydride and distilled under nitrogen) β-mercaptoethanol (this would be expected from reaction 2), as well as by ordinary β-mercaptoethanol (this would not be expected from reaction 1, assuming the thiol solution already contained disulfide).

In the many experiments of the sort illustrated by Figures 5 and 6 the system with maximal ATPase activity during modification has an activity very near that of the system attained by regenerating the 24-hour aliquot; however, the former contains not less than[2] 1.2 moles SH/10^5 g myosin, while the latter contains 0.16 mole SH/10^5 g myosin. There are two ways of interpreting such a result, depending on how one looks at the relationship between the X → X′ and α → β processes, and the Y → Y′ and β → γ processes. Rainford *et al.* (1964) and Sekine *et al.* (1962) have argued that, since reagents with no obvious affinity for SH (i.e., reagents which probably cannot achieve X → X′, Y → Y′, or Z → Z′) nonetheless cause α → β and β → γ, the enzymatic modification processes, α → β and β → γ, must be processes distinct from, and subsequent to, the sulfhydryl reactions. If so, then the reversal of the SH reactions may or may not cause the reversal of the modifications. On the other hand, X → X′ and α → β may be one and the same reaction, as may Y → Y′ and β → γ; in this case there is a unique distribution of α, β, and γ states corresponding to each distribution of X′, Y′, and Z′. According to the first, and more likely, hypothesis the systems reached by modification to maximal activity and by regeneration to maximal activity are, or could be, exactly alike as regards distribution of α, β, and γ states among the enzyme molecules, but the latter system has much less bound thiopropionate because thiopropionate can be stripped from the enzyme without necessarily reversing the activity effects caused when the thiopropionate was originally attached. But our experiments do not *logically* exclude an alternate explanation, viz., that the distribution of α, β, and γ states (and therefore of X′, Y′, and Z′) reached on regeneration is quite different from that reached on initial modification; different in being more "efficient," for example, in

[2] With our technique modification is not instantly stopped so it is hard to catch precisely maximal ATPase activity. Also, because the initial rate of modification is very fast, even the "zero"-time aliquot already has appreciable bound radioactivity (1.2 moles SH/10^5 g myosin). This initial radioactivity has been subtracted from the 2.6 moles SH/10^5 g myosin actually measured for the 24-hour aliquot to obtain the lower limit cited.

containing much greater ratios of $\beta:\alpha$ and $\beta:\gamma$, therefore giving the same V (max) with a lesser $\bar{n}$.

We leave quite unexplained two other features of the regeneration experiments: (a) The same regeneration treatment which returns optimally modified myosin to control levels as regards $\bar{V}$ (max) and $\bar{n}$ fails with myosin *regenerated* to maximal $\bar{V}$ (max). (b) Aliquots of enzyme which has been modified for *ca.* 50 hours cling more effectively to their bound thiopropionate than aliquots incubated for shorter or longer times (Figure 5a).

Generally, our results confirm and amplify the various techniques used by Stracher (1963, 1964); but, quantitatively, we meet some difficulties of correlation. Taking 6×10^5 g as the molecular weight of myosin (see, however, footnote 1), and 7.5 moles/10^5 g as its SH content, Stracher finds that after 96 hours of incubation with dithiopropionate (conditions as in 200-fold excess case, Figure 3) myosin loses its ATPase activity, and that each mole of myosin can bind 3 moles of (^{14}C-labeled) iodoacetamide; from this he calculates that before iodoacetamide treatment each mole of myosin must have contained 3 moles of free SH (0.456 mole SH/10^5 g myosin). Stracher also finds that, before iodoacetamide treatment, dithiopropionated myosin, regenerated by incubating 16 hours with 200-fold molar excess of β-mercaptoethanol, fully recovers its ATPase activity, but after iodoacetamide treatment regeneration becomes impossible. Assuming that myosin consists of three equivalent strands (and, presumably, also assuming that it has three ATPase sites), Stracher concludes that iodoacetamide labels an SH group at the active site. We have no indication that any SH of myosin is invulnerable to disulfide exchange (indeed, the concentration dependence shown in Figure 3 and the inability to detect *p*-mercuribenzoate binding after, say, 168 hours of exchange argue to the contrary); moreover, our myosin preparations, titrated spectrophotometrically, vary in SH content (e.g., 6.8–8.5 moles SH/10^5 g myosin). Therefore, if our preparations were exposed to a standard incubation with dithiopropionate they would undoubtedly retain variable amounts of free SH, and an iodoacetamide label would be neither constant in amount nor uniquely placed. Figure 5a shows another slight discrepancy with Stracher's work. In our experience, aliquots modified for 75–125 hours are regenerated by 3-mercaptopropionate to ATPase activity levels roughly like those of control enzyme (actually 20–30% higher), but from the shape of the entire curve of regenerated activity, it is clear that the similarity is fortuitous. We are thus not in a position to say that the ATPase activity lost through incubation for 96 hours is precisely regained on regeneration, especially not if β-mercaptoethanol is the regenerating agent (Figure 1). Lastly we must remark that, assuming Stracher's interpretation, one must also assume that reaction of SH groups different from the iodoacetamide-labeled group must somehow destroy the active site, since he reports ATPase activity to be zero *before* iodoacetamide treatment.

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