indicated by the experiments with the truncated aromatic analogues of Cibacon Blue, Procion Blue MX-R, and anthraquinonesulfonic acid.

In conclusion, myosin subfragment 1 contains multiple Cibacron Blue binding sites. The binding of S-1 to the immobilized dye does not involve the protein's active site. The reversible inhibition of the ATPase activity of S-1 is related to the binding of Cibacron Blue to a single site on S-1 which appears to be distinct from the nucleotide binding site.

In a more general sense, our results underscore the complexity of protein–dye interactions and point out the risks inherent in extrapolating from the properties and behavior of one protein to other proteins. Protein binding to immobilized dyes and inhibition of catalytic activity need not necessarily imply the direct involvement of the nucleotide site in these events, let alone serve as a basis for structural conjectures.

References

Ashton, A. R., & Polya, G. M. (1978) *Biochem. J. 175*, 601–607.

Beissner, R. S., & Rudolph, F. B. (1978) *Arch. Biochem. Biophys. 189*, 76–80.

Beissner, R. S., Quiocho, F.-A., & Rudolph, F. B. (1979) *J. Mol. Biol. 134*, 847–850.

Burke, M., & Reisler, E. (1977) *Biochemistry 16*, 5559–5563.

Chambers, B. B., & Dunlap, R. B. (1979) *J. Biol. Chem. 254*, 6515–6521.

Dean, P. D. G., & Watson, D. S. (1979) *J. Chromatogr. 165*, 301–319.

Drocourt, J. L., Thank, D.-C., & Thang, M.-N. (1978) *Eur. J. Biochem. 82*, 355–362.

Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375–400.

Godfrey, J., & Harrington, W. F. (1970) *Biochemistry 9*, 886–892.

Jankowski, W. J., Muenchhausen, E., Sulkowski, E., & Carter, W. A. (1976) *Biochemistry 15*, 5182–5188.

Kielley, W. W., Kalckar, H. M., & Bradley, L. B. (1956) *J. Biol. Chem. 219*, 95–112.

Kumar, S. A., Beach, T. A., & Dickerman, H. W. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2199–2203.

Leatherbarrow, R. J., & Dean, P. D. G. (1980) *Biochem. J. 189*, 27–34.

Panefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.

Reisler, E., Liu, J., Mercola, M., & Horwitz, J. (1980) *Biochim. Biophys. Acta 623*, 243–256.

Seelig, G. F., & Colman, R. F. (1979) *J. Biol. Chem. 252*, 3671–3678.

Stellwagen, E. (1977) *Acc. Chem. Res. 10*, 92–98.

Thompson, S. T., & Stellwagen, E. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 361–365.

Thompson, S. T., Cass, K. H., & Stellwagen, E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 669–672.

Toste, A. P., & Cooke, R. (1979) *Anal. Biochem. 95*, 317–328.

Travis, J., & Pannell, R. (1973) *Clin. Chim. Acta 49*, 49–52.

Weber, B. H., Willeford, K., Moc, J. G., & Piszkiewicz, D. (1979) *Biochem. Biophys. Res. Commun. 86*, 252–258.

Weeds, A.-G., & Taylor, R. S. (1975) *Nature (London) 257*, 54–56.

Wells, J. A., & Yount, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966–4970.

Wells, J. A., Sheldon, M., & Yount, R. G. (1980) *J. Biol. Chem. 255*, 1598–1602.

Witt, J. J., & Roskoski, R., Jr. (1980) *Biochemistry 19*, 143–148.

Ionization of Reactive Lysyl Residue of Myosin Subfragment 1†

Andras Muhlrad*,‡ and Reiji Takashi

ABSTRACT: The ϵ-NH_2 groups of lysyl residues of myosin subfragment 1 belong to two classes on the basis of their reaction with 2,4,6-trinitrobenzenesulfonate: one reactive lysyl residue and 82 slow-reacting lysyl residues. The trinitrophenylation of the reactive lysyl residue is accompanied by a sharp decrease in the K^+(EDTA)-activated ATPase of myosin subfragment 1. The rate of trinitrophenylation of this group was followed as an increase in A_{345} or as a decrease in K^+(EDTA)-activated ATPase at various pHs between 6.5 and 10. The second-order rate constant obtained by these methods sharply increased with pH, plateauing at about pH 9.7. A typical dissociation curve with pK = 9.0 was obtained by plotting the pH dependence of the rate constant. For this reactive lysyl, the pK value was low and the maximal rate of trinitrophenylation was high in comparison to the corresponding quantities of the slow-reacting lysyls of myosin subfragment 1 and of a model compound, N^{α}-carbobenzoxy-L-lysine. The pH dependence of the trinitrophenylation of lysyl residues of myosin subfragment 1 was anomalous. The pK value and maximal rate of trinitrophenylation of poly-L-lysine resembled those of the reactive lysyl residue. The presence of an aromatic moiety in the model compound was found to promote trinitrophenylation. It is suggested that the anomalous behavior of the reactive lysyl residue is caused by a vicinal positive charge and by other neighboring groups.

Myosin subfragment 1 (S-1)[1] is the segment of the myosin molecule responsible for ATPase activity and interaction with actin. Well-defined functional groups, such as reactive thiols, reactive lysine, certain tryptophanes, are located in this segment. The modification of these residues characteristically affects the activities of myosin. One of these functional groups is a reactive lysyl residue (RLR) described first on the basis

† From the University of California, San Francisco, Cardiovascular Research Institute, San Francisco, California 94143. *Received November 14, 1980; revised manuscript received June 23, 1981.* This research was supported by Grants HL-16683, PCM 75-22698 (National Science Foundation), and AHA CI-8.

‡ Senior investigator of the Muscular Dystrophy Association. Present address: Department of Oral Biology, Hebrew University, Hadassah School of Dental Medicine, Jerusalem, Israel.

[1] Abbreviations used: S-1, myosin subfragment 1; TNBS, 2,4,6-trinitrobenzenesulfonate; TNP, trinitrophenyl; RLR, reactive lysyl residue; N^{α}-Cbz-lysine, N^{α}-carbobenzoxy-L-lysine.

0006-2960/81/0420-6749$01.25/0

of its reaction with TNBS by Kubo et al. (1960), who found two RLRs per myosin. The modification of RLR was accompanied by a marked increase in the Mg^{2+}-activated ATPase activity (Kitagawa et al., 1961) and decrease in the K^+(EDTA)-activated (Fabian & Muhlrad, 1968) ATPase activity of myosin. The RLR was located in the heavy chain of myosin (Muhlrad & Afolayan, 1975), more specifically in the 27 000 fragment of S-1 (Miyanishi, 1980; Hozumi & Muhlrad, 1981). There is one RLR per S-1 (Muhlrad et al., 1975). The RLR is generally defined by its reaction with 2,4,6-trinitrobenzenesulfonate (TNBS) leading to the trinitrophenylation of the residue, a reaction that can be easily followed spectrophotometrically. The reaction occurs in a fast and then a slow phase. RLR is trinitrophenylated in the fast phase while the rest of the lysyl residues react slowly in both phases of the reaction. Both reactions were characterized by second-order rate constants; that of RLR is 3 orders of magnitude greater than that for the slowly reacting residues (Muhlrad et al., 1975, 1976). It seemed of interest to characterize further the trinitrophenylation of RLR by measuring the dependence of the rate of the reaction on pH. This might give some insight into the ionization of RLR and thus explain its unusually high reactivity, which is one of the unique features of myosin S-1.

Materials and Methods

Chemicals. N^{α}-Cbz-lysine, N^{α}-acetyl-L-lysine, N^{α}-acetyllysine methyl ester, poly-L-lysine and ATP were from Sigma Chemical Co., and TNBS was an Aldrich Chemical Co. product. All other chemicals were of reagent grade.

Proteins. Myosin from back and leg muscles of rabbit was prepared by the method of Tonomura et al. (1966). S-1 was prepared by digestion of myosin filaments with chymotrypsin (Weeds & Taylor, 1975) and purified by filtration through Sephacryl S-200 in 20 mM Tes, pH 7.0.

Protein Concentration. S-1 concentration was calculated from its absorbance, assuming an absorption coefficient, $A^{1\%}_{1cm,280nm}$ of 7.5. The molecular weight of S-1 was assumed to be 110 000.

Trinitrophenylation of S-1 and of Model Compounds. The kinetics of trinitrophenylation was followed spectrophotometrically essentially as described earlier (Muhlrad et al., 1975). TNBS (final concentration 100–125 μM) was added to a solution containing 8–10 μM S-1 or 1.0 mM model compound in 100 mM buffer; in the case of poly-L-lysine, 1 mM was the concentration of the lysine monomer. The reaction was carried out in a thermostated cell (25 °C) of a Cary 118C spectrophotometer, and the absorbance change at 345 nm was recorded. This absorbance change was used to evaluate the number of lysyl residues trinitrophenylated by assuming $\Delta\epsilon_{345}$ = 14 500 (Okuyama & Satake, 1960). In the range of pH 6–11.5, pH did not affect the extinction coefficient of TNP-lysine, but a 5% decrease in the extinction coefficient was observed between pH 11.5 and pH 12.0.

ATPase Assay. The effect of trinitrophenylation on the K^+(EDTA)-activated ATPase of S-1 was studied. In due time 25-μL aliquots were withdrawn from the trinitrophenylation reaction mixture described in the previous paragraph and their ATPase activity was measured immediately. The aliquots were diluted 40-fold in the ATPase assay mixture containing 2 mM ATP, 600 mM KCl, 5 mM EDTA, and 50 mM Tris-HCl, pH 8.0. The dilution induced a 1600-fold deceleration of the trinitrophenylation reaction, considering that trinitrophenylation of S-1 is a second-order reaction (Muhlrad et al., 1975), in which the velocity of the reaction is proportional to the concentrations of both reactants. This assured that the ATPase activity measured was characteristic of the sample at the time it was withdrawn from the trinitrophenylation reaction mixture. ATP hydrolysis proceeded for 3 min at 25 °C, and then the reaction was stopped by addition of sulfuric acid containing ammonium molybdate and the P_i content was determined by the method of Fiske and Subbarow (1929). Never more than 15% of the ATP was hydrolyzed. ATPase activity was expressed as

$$\text{micromoles of } P_i \times (\text{milligrams of S-1})^{-1} \times \text{minute}^{-1}$$

pH was measured at the beginning and the end of the trinitrophenylation reaction by a Radiometer Model 26 pH meter.

Electrometric titrations were performed in a Radiometer REC 61/REA 160 recording titration system on 10 mM model compound in 150 mM KCl with 0.5 N NaOH. The titration curves were corrected for the alkali uptake of the medium.

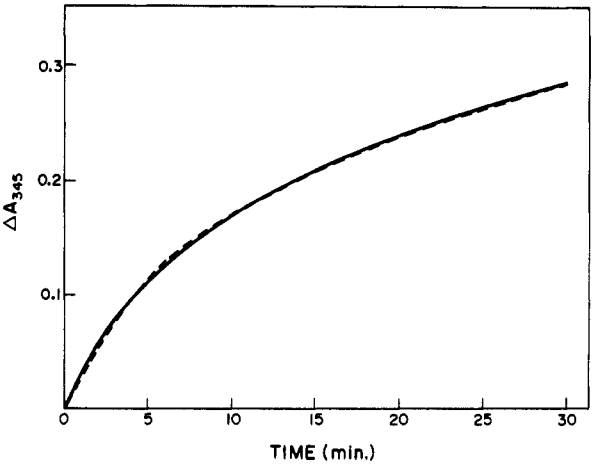

FIGURE 1: Kinetics of trinitrophenylation of S-1. 0.1 mM TNBS was added to 10 μM S-1 in 100 mM imidazole, pH 7.37, 25 °C, and the change in A_{345} was recorded. Experimental curve (—); computer-simulated curve (---) drawn by using the calculated kinetic constants.

Results

The trinitrophenylation of S-1 was followed spectrophotometrically (Figure 1). The chemical equation was assumed to be

$$-NH_2 + TNBS \rightarrow -NH\text{-}TNP + HSO_3^- + H^+$$

However, we further assumed that per S-1 [at C_0 (moles per liter)] there were m lysyls reacting quickly [k_f (min^{-1} M^{-1})] and n lysyls reacting slowly [k_s (min^{-1} M^{-1})], with a total [TNBS] = T_0. If u and w, respectively, are the concentrations of "fast" and "slow" lysyls reacted after time t, then

$$du/dt = k_f(mC_0 - u)(T_0 - u - w) \quad (1)$$

$$dw/dt = k_s(nC_0 - w)(T_0 - u - w) \quad (2)$$

describe the simultaneous reactions. Equations 1 and 2 were simulated on a computer by using various values of k_f, k_s, m, n, and we chose the best fitting parameter set by a least-squares method. For the data of Figure 1, 1660, 4, 1, and 82 were obtained, respectively.[2]

[2] We also checked whether it is possible to obtain an even better fit by assuming other sets of lysyl residues with intermediate reactivity. However, best fit was observed only by assuming identical reactivity for all 82 "slow" lysyls.

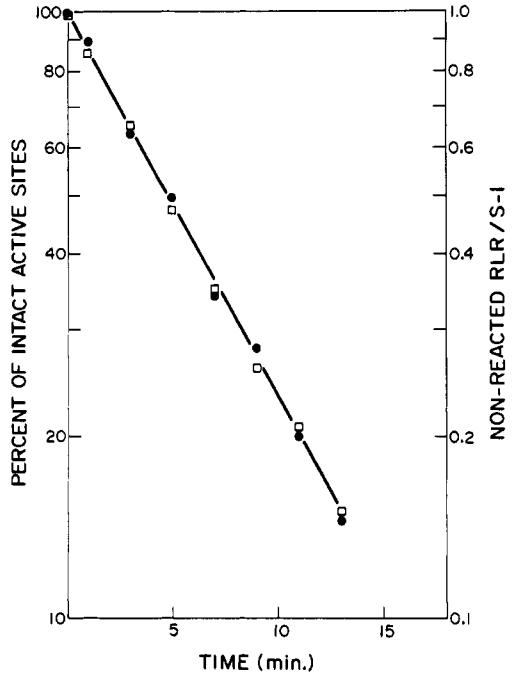

FIGURE 2: Decrease in intact active sites and unreacted RLR of S-1 during trinitrophenylation. S-1 was trinitrophenylated as described in Figure 1. Nonreacted RLR (□) was calculated by using the kinetic constants obtained from the computer analysis of Figure 1. Intact active site (●) values were calculated from the ATPase data.

The effect of trinitrophenylation on K^+(EDTA)-activated ATPase of S-1 was also studied under the same conditions. ATPase activity decreased sharply, reaching a plateau 13 min after addition of TNBS. The activity at the plateau was about 10% of that of the unmodified S-1 and was assumed to be characteristic of trinitrophenylated S-1. Thus, the percentage of intact (unaffected) active sites could be evaluated at intermediate times. The logarithm of the percentage of active sites was plotted as a function of time (Figure 2) and linear dependence was obtained. Thus the reaction was pseudo first order with respect to active sites, provided that the concentration of TNBS remained essentially constant during the experiment. This proviso was met, as TNBS concentration decreased less than 10% during the first 13 min of the reaction. It was shown earlier (Muhlrad et al., 1975) that the rate of the loss of activity depended on TNBS concentration. The decrease in the nonreacted RLR during the same period was computed by using the rate constant k_f obtained above. When the logarithm of nonreacted RLR/S-1 was plotted against time (Figure 2), virtually the same linear dependence was obtained as with the active sites, showing that the trinitrophenylation of RLR was responsible for the loss of activity. As the second-order rate constant, k_{enz}, computed from the loss of enzymatic activity was virtually equal with k_f, the measurement of k_{enz}, provided us with an independent method for the evaluation of the rate of trinitrophenylation of RLR.

S-1 was trinitrophenylated at various pHs between 6.5 and 10, and k_f, k_{enz} (Figure 3), and k_s (Figure 4) values were evaluated as above. The k_f and k_{enz} values were very close to each other at every pH studied; they increased with increasing pH, generating ionization curves. The maximum values of k_f and k_{enz} were of the order of 3×10^4 min^{-1} M^{-1}. The pH at the half-maximal value, which was considered to be equal to the pK of the RLR, was 9.04.

However, the ionization curve was asymmetrical; the pH at half-maximum (9.04) was not equal to the pH at the inflection (9.2). The true pK can therefore not be accurately estimated from Figure 3. The asymmetry may be due to

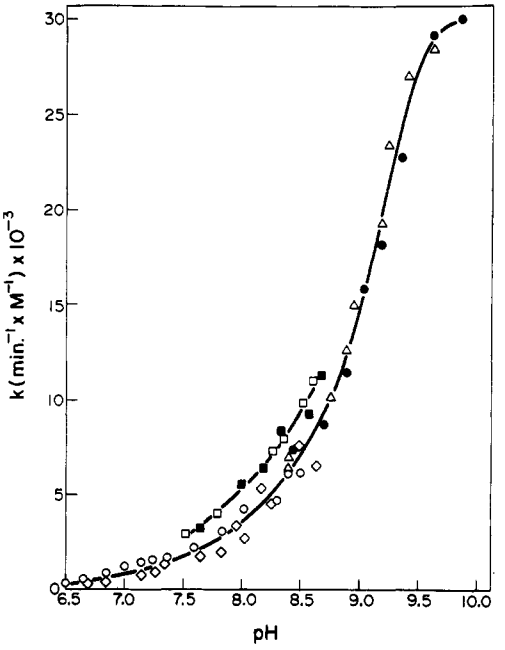

FIGURE 3: pH dependence on the rate of trinitrophenylation of RLR. Rate constants were obtained from either computer analysis of spectrophotometer curves, k_f (○; □; △), or from ATPase activity assay, k_{enz} (◇; ■; ●), in the presence of 100 mM imidazole (○; ◇), Tris-HCl (□; ■), or borate (△; ●) buffer 25 °C.

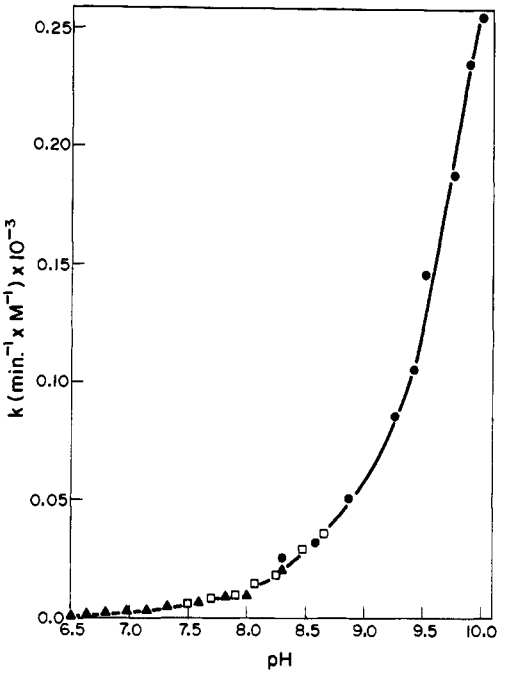

FIGURE 4: pH dependence of the rate of trinitrophenylation of slow-reacting lysyl residues in S-1. Rate constants, k_s, were obtained from computer analysis of spectrophotometer curves in the presence of 100 mM imidazole (▲), Tris-HCl (□), or borate (●) buffer, 25 °C.

suppression of the reaction at high pH.[3] The k_f and k_{enz} values were identical whether the buffer was borate or imidazole. However, higher values of these constants were obtained by using Tris buffer. The k_s values also increased with increasing

[3] At high pH trinitrophenylation of S-1 was very fast, so measurement in 3–4 min was feasible. During this period the K^+(EDTA)-activated ATPase of the nontrinitrophenylated (control) enzyme was stable at pH ≤10. Also, it was found that enzyme first exposed to pH 9.8 (5 min at 25 °C) and then returned to pH 7.7 produced the same parameters as enzyme not thus exposed. Any "denaturation" produced at high pH must therefore be reversible.

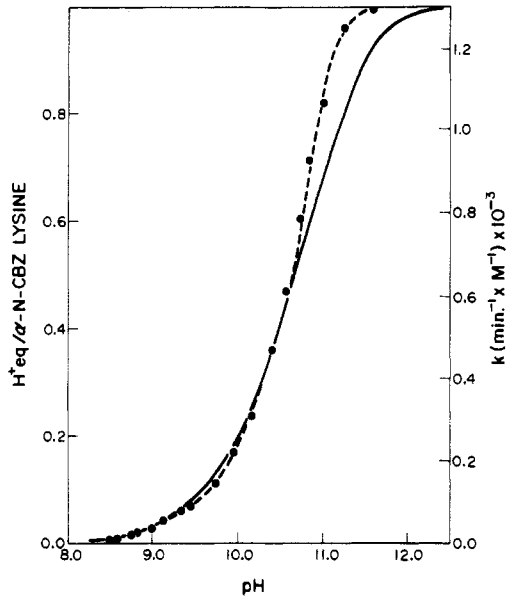

FIGURE 5: pH dependence of the rate of trinitrophenylation and electrometric titration of N^{α}-Cbz-lysine. Rate constants of trinitrophenylation (---) were assessed from spectrophotometer curves; 1 mM N^{α}-Cbz-lysine; 0.1 mM TNBS; 100 mM borate buffer, 25 °C. Electrometric titration (—) of 10 mM N^{α}-Cbz-lysine in 0.15 KCl was carried out with 0.5 N NaOH at 25 °C. Electrometric titration was also carried out in the absence of KCl, and virtually the same curve was obtained as in the presence of salt.

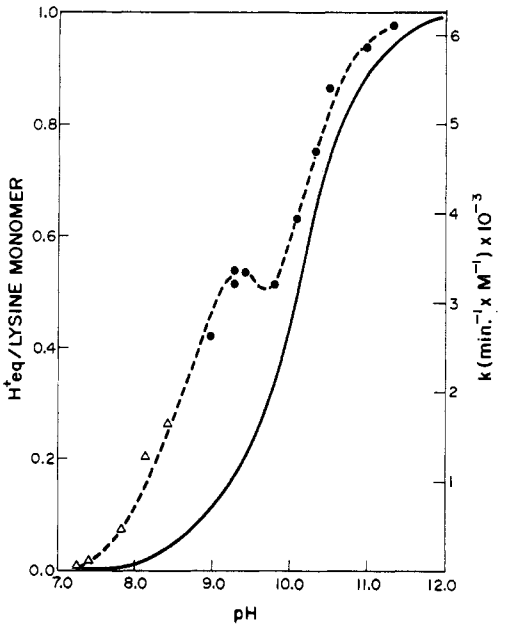

FIGURE 6: pH dependence of the rate of trinitrophenylation and electrometric titration of poly-L-lysine. Rate constants of trinitrophenylation (---) were assessed from spectrophotometer curves; monomer concentration of 1 mM poly-L-lysine; 0.1 mM TNBS; 100 mM imidazole (Δ) or borate (●) buffer, 25 °C. Electrometric titration (—) of 10 mM poly-L-lysine (on the basis of lysine monomer) in 0.15 M KCl was carried out with 0.5 N NaOH at 25 °C. Electrometric titration was also carried out in the absence of KCl, and virtually the same curve was obtained as in the presence of salt.

pH, but no plateau was reached by pH 10, the experimental upper limit due to enzyme denaturation. Because a plateau of k_s was unattainable, it was impossible to estimate a pK for the slowly reacting lysyl residues, but it is obvious that it is considerably higher (perhaps by 1 pH unit) than that of the RLR.

In order to learn more about the ionization of the RLR, we also studied the pH dependence of trinitrophenylation of model compounds. The trinitrophenylation of N^{α}-Cbz-lysine was followed spectrophotometrically. A straight line was recorded, provided that the TNBS and model compound concentration remained constant during the measurement (5 min). The second-order rate constant of the reaction was calculated from the slope. The pH dependence of the rate constants (Figure 5) had the shape of an ionization curve with a maximum value of 1300 min^{-1} M^{-1} and pK of 10.7. Electrometric titration was also performed on the model compound (Figure 5), and the resulting ionization curve was very similar to the curve obtained on measuring the pH dependence of rate of trinitrophenylation, especially at pH values lower than the pK of the ϵ-NH_2 group. The pK of this model compound was higher and the maximal value of the rate constant was much lower than the respective values of RLR.

It was assumed that anomalously low pK and high maximal rate constant of RLR might be the result of a vicinal positive charge. In order to check this possiblity, we used poly-L-lysine as a model because of the density of positive charge bearing lysyl residues in this compound. The second-order rate constant of trinitrophenylation of polylysine was computed as described in the previous paragraph. The pH dependence of the rate constant (Figure 6) resembled that of RLR in the sense that a considerably greater ionization value was obtained at low pH. The peculiarity of the curve was a trough at pH 9.6. The trough could be the result of a helix–coil transition in the structure of polylysine which takes place at this pH (Applequist & Doty, 1962; Tseng & Yang, 1977). A pK could not be computed because of the trough and the maximal value of the rate constant was 6000 min^{-1} M^{-1}. However, the pK, 10.1, could be calculated from the electrometric titration curve which was smooth and considerably different from the trinitrophenylation curve, especially at low pHs.

Table I: Rate Constants of Trinitrophenylation of the ϵ-NH_2 Group of Lysine in S-1 and Model Compounds at pH 9.54[a]

	k (min^{-1} M^{-1})
N^{α}-Cbz-lysine	66.3
N^{α}-acetyl-L-lysine	46.6
N^{α}-acetyl-L-lysine methyl ester	83.6
poly-L-lysine	3 365
S-1, k_f	27 200
S-1, k_{enz}	27 080
S-1, k_s	132

[a] 1 mM model compound (1 mM lysine monomer in the case of poly-L-lysine) or 10 μM S-1 was reacted with 0.1 mM TNBS in the presence of 100 mM sodium borate, pH 9.54, at 25 °C, and the change in A_{345} or in K^+ (EDTA)-activated ATPase (S-1, k_{enz}) was recorded as described under Materials and Methods.

The effect of various vicinal groups on the rate of trinitrophenylation of lysine ϵ-NH_2 group was further studied by comparing the trinitrophenylation of different model compounds at pH 9.54 in the presence of borate buffer (Table I). The results show that the vicinity of a carboxyl group bearing negative charge decreases the rate of trinitrophenylation (N^{α}-acetyl-L-lysine vs. N^{α}-acetyl-L-lysine methyl ester) while an aromatic group in the neighborhood increases the rate of the reaction (N^{α}-Cbz-lysine vs. N^{α}-acetyl-L-lysine). This is in addition to the already observed effect of vicinal positive charge (polylysine).

Discussion

The reactivity of the RLR of S-1 toward TNBS between pH 7 and pH 8 is almost 3 orders of magnitude greater than that of the other lysyl residues of S-1. It is very probable that a factor affecting the reactivity of the RLR is a vicinal positive charge, as was suggested for a reactive lysine of ribonuclease by Hirs (1962). A neighboring cationic center could labilize

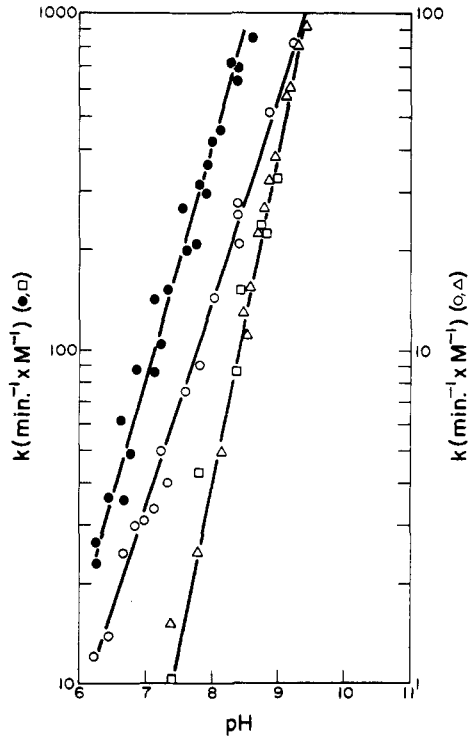

FIGURE 7: pH dependence of logarithm of rate constants of trinitrophenylation of S-1 and model compounds. Rate constants of S-1, k_f (●) and k_s (○); rate constants of model compounds, poly-L-lysine (□) and N^{α}-Cbz-lysine (△). Data were obtained from Figures 3–6. The lines were calculated by the least-squares method. Left-hand-side ordinate is for k_f of S-1 and poly-L-lysine; right-hand-side ordinate is for k_s of S-1 and N^{α}-Cbz-lysine.

the proton of the amino group by repulsion and effecively decrease its pK. This seems to be happening in polylysine where the amino groups have a higher reactivity toward TNBS and a lower pK than does the ϵ-amino group of N^{α}-Cbz-lysine. However, a positive charge vicinal to RLR does not seem to be sufficient explanation for the high reactivity as the intrinsic rate constant of the RLR is still considerably higher and its pK is lower than the respective values of polylysine. A factor which may contribute to the increased reactivity could be an aromatic group which was shown to increase the reactivity of the model compound (Table I). According to our recent observations (T. Hozumi and A. Muhlrad, unpublished experiments) the 27 000 fragment of S-1, where the RLR located is also rather rich in tryptophan, which may enhance the reactivity of the RLR. A thiol group may also affect RLR reactivity. It was found that the SH_1 thiol was located near to RLR and the blocking of the SH_1 group decreases the reactivity of RLR and increases its pK (Takashi et al., 1980).

Another interesting question is how unique is this high reactivity in comparison with that of reactive lysyl residues in other proteins? Trinitrophenylation of a number of proteins has been studied: human serum albumin (Goldfarb, 1966b), different trypsin inhibitors (Haynes et al., 1967), chymotrypsinogen, ribonuclease, insulin, glutamic dehydrogenase (Freedman & Radda, 1968), actin (Muhlrad, 1968), growth hormone (Cascone et al., 1977), dynein (Shimizu, 1979), etc. High reactivity of ϵ-amino groups has been reported in several cases but never as high as that of the RLR of S-1. The RLR seems to be a characteristic functional group of the myosin head; it is present in all myosins (fast and slow skeletal, cardiac and smooth muscle, and blood platelets) in which the trinitrophenylation reaction has been studied so far (Muhlrad et al., 1976; Srivastava et al., 1979). The reactivity of RLR is highest in fast skeletal muscle myosin. As the modification of RLR is coupled to profound changes in the enzymatic activity of myosin, this group might have a functional role in a sensitive part of the molecule.

A practical question for functional studies is how specific is the trinitrophenylation of RLR relative to that of the slow-reacting lysyls of S-1? It was found that Tris buffer enhances the reactivity of the RLR without affecting that of slow-reacting lysyls. According to our computer simulations, in the presence of Tris, pH 7.7, 70% of the label attached to RLR when the average number of TNP-lysine formed per S-1 was one.

Another feature of the trinitrophenylation of RLR, and also of slow-reacting lysyls of S-1, is an anomalous pH dependence. It is anomalous in two senses: the ionization curve is asymmetrical because of the suppression of the reaction at high pH, and the slopes of log k vs. pH plots are less than unity at low pH (see Appendix). At low pH, when the measured rate constant is proportional to the fraction of the unprotonated form, these slopes should be unity. The latter anomaly was not observed in poly-L-lysine where a normal pH dependence was found at low pH. Therefore, the reason for this anomaly cannot be a vicinal positive charge. Goldfarb (1966b) observed the same phenomenon in human serum albumin. His explanation was that trinitrophenylation might be a complex reaction where the velocity of the first step, i.e., the association of TNBS and the amino group, would depend on the concentration of the unprotonated form. However, the rate-determining step would be the second step in which the actual trinitrophenylation occurs and this step can have a different pH dependence from the first one. However, an additional or alternative explanation might exist. Trinitrophenylation of macromolecules greatly depends on the conformation of the compound, as it was shown in the case of poly-L-lysine where the rate of the reaction depended on the helix–coil transition (Figure 6) (Applequist & Doty, 1962; Tseng & Yang, 1977). It might be that the increasing pH induces generalized conformational changes in S-1 (or serum albumin) and this alters the reactivity of lysyls toward TNBS independent of the change in ionization and causes the anomalous pH dependence. The structural sensitivity to trinitrophenylation may explain the buffer and ionic strength dependence of the reaction (A. Muhlrad, unpublished observations) and the reduced rate of trinitrophenylation of RLR in the presence of MgATP and its analogues (Tonomura et al., 1963; Muhlrad & Fabian, 1970).

In summary, the ionization of the reactive lysyl residue of S-1 has been studied by means of its TNBS reaction. It has an unusually low pK and an anomalous pH dependence. Its high reactivity toward TNBS may be caused by the environment, e.g., by the vicinity of positive charge or of aromatic moieties or of SH groups. The trinitrophenylation of lysyl residues in general and RLR in particular can be a useful tool in studying subtle conformational changes in myosin because of high structural sensitivity of the reaction.

Acknowledgments

We are indebted to Professor Manuel F. Morales for valuable discussions during the experiments and preparation of the manuscript and to Dr. Julian Borejdo for his kind help in preparation of the computer programs.

Appendix

A plot of the trinitrophenylation rate constant vs. pH resembles an ionization curve and suggests that the reaction occurs only with the unprotonated form of the reactive ϵ-NH_2 [cf. Goldfarb (1966a)]. If so, the rate constant at a particular pH should be k_i, an intrinsic rate constant (the highest ob-

served), multiplied by y, the fraction of the ϵ-NH_2 that is unprotonated. It is easy to show that $y = 1/[1 + \exp[a(pK - pH)]]$, where $a = 2.303....$ When pH is much less than pK [i.e., $a(pK - pH)$ is large], the exponential is much larger than unity, and $k \simeq k_i \exp[a(pH - pK)]$, whereupon

$$\log k = pH - (pK - \log k_i)$$

Since in the enzyme cases k_i is not precisely known (the plateau value), pK cannot be obtained from the intercept of this plot, but when log k is plotted against pH, a straight line of slope unity should be obtained. This was exactly so for N^{α}-Cbz-lysine and poly-L-lysine; for k_f and k_s, however, slopes of 0.7 and 0.61, respectively, were obtained (Figure 7). There is thus a further anomaly in the ionization curves of the enzyme ϵ-NH_2. In the case of the model compounds, the plateau levels could be measured, and the intercepts gave pK - log k_i values of 7.4 and 6.4, respectively—in good aggrement with estimates from Figures 5 and 6.

References

Applequist, J., & Doty, P. (1962) in *Polyamino Acids, Polypeptides and Proteins* (Stahmann, M. A., Ed.) p 161, University of Wisconsin Press, Madison, WI.

Cascone, O., Bonino, B. J. M., Paladini, A. C., & Santome, J. A. (1977) *Arch Biochem. Biophys. 180*, 303-309.

Fabian, F., & Muhlrad, A. (1968) *Biochim. Biophys. Acta 162*, 596-603.

Fiske, C. H., & Subbarow, Y. (1929) *J. Biol. Chem. 66*, 375-400.

Freedman, R. B., & Radda, G. K. (1968) *Biochem. J. 108*, 383-391.

Goldfarb, A. R. (1966a) *Biochemistry 5*, 2570-2574.

Goldfarb, A. R. (1966b) *Biochemistry 5*, 2574-2578.

Haynes, R., Osuga, D. T., & Feeney, R. E. (1967) *Biochemistry 6*, 541-547.

Hirs, C. H. W. (1962) *Brookhaven Symp. Biol. 15*, 154-183.

Hozumi, T., & Muhlrad, A. (1981) *Biochemistry 20*, 2945-2950.

Kitagawa, S., Yoshimura, J., & Tonomura, Y. (1961) *J. Biol. Chem. 236*, 902-906.

Kubo, S. Tokura, S., & Tonomura, Y. (1960) *J. Biol. Chem. 233*, 2835-2839.

Miyanishi, T. (1980) Japan-U.S. Seminar, Sapporo, Japan.

Muhlrad, A. (1968) *Biochim. Biophys. Acta 162*, 444-451.

Muhlrad, A., & Fabian, F. (1970) *Biochim. Biophys. Acta 216*, 422-427.

Muhlrad, A., & Afolayan, A. (1975) *J. Mechanochem. Cell Motil. 3*, 99-101.

Muhlrad, A., Lamed, R. R., & Oplatka, A. (1975) *J. Biol. Chem. 250*, 175-181.

Muhlrad, A., Oplatka, A., & Lamed, R. (1976) *Biochim. Biophys. Acta 452*, 227-238.

Okuyama, T., & Satake, K. (1960) *J. Biochem. (Tokyo) 47*, 454-466.

Shimizu, T. (1979) *J. Biochem. (Tokyo) 85*, 1421-1426.

Srivastava, S. K., Tonomura, Y., & Inoue, A. (1979) *J. Biochem. (Tokyo) 86*, 725-31.

Takashi, R., Muhlrad, A., Hozumi, T., & Botts, J. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1936.

Tonomura, Y., Yoshimura, J., & Onishi, T. (1963) *Biochim. Biophys. Acta 78*, 698-704.

Tonomura, Y., Appel, P., & Morales, M. F. (1966) *Biochemistry 5*, 515-521.

Tseng, Y. W., & Yang, J. T. (1977) *Biopolymers 16*, 921-935.

Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54-56.

Synthesis and Application of Cleavable Photoactivable Heterobifunctional Reagents†

Elio F. Vanin and Tae H. Ji*

ABSTRACT: Three different cleavable photoactivable heterobifunctional reagents have been synthesized and characterized by proton NMR, ^{13}C NMR, mass spectroscopy, infrared spectroscopy, and thin-layer chromatography. They are the *N*-hydroxysuccinimide ester of 3-[(4-azidophenyl)dithio]-propionic acid, methyl 3-[(4-azidophenyl)dithio]propionimidate, and *N*-[(4-azidophenyl)thio]phthalimide. Concanavalin A was coupled with one of the reagents, the *N*-hydroxysuccinimide ester of 3-[(4-azidophenyl)dithio]propionic acid, and radioiodinated. The [^{125}I]lectin derivative and [^{125}I]lectin showed similar specific binding to the receptors on the human erythrocyte membrane. Upon photolysis, subunits of the [^{125}I]lectin derivative were cross-linked to produce dimer, trimer, and tetramer. These cross-linked complexes were readily cleaved by reducing the disulfide bond of the reagent.

Chemical cross-linking has been a major tool for predicting subunit structures of molecules and molecular associations in cell membranes [for reviews, see Peters & Richards (1977) and Ji (1979)]. Recently its application has been extended to the identification of surface receptors for macromolecular ligands (Ji, 1976). In this case, photoactivable heterobifunctional reagents are coupled to macromolecular ligands via the nonphotosensitive chemically reactive group, and the ligand derivative is cross-linked to receptors by photoactivation after binding. Therefore, these reagents can be used effectively to probe membrane receptors and their environment in intact cells.

To date most of the available photoactivable heterobifunctional reagents used for receptor studies are noncleavable.

† From the Department of Biochemistry, University of Wyoming, Laramie, Wyoming 82071. *Received May 18, 1981.* This work was supported by Grant GM-23586 from the National Institutes of Health.

0006-2960/81/0420-6754$01.25/0