Cross-Linking of Three Heavy-Chain Domains of Myosin Adenosinetriphosphatase with a Trifunctional Alkylating Reagent

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ABSTRACT: The chemotherapeutic alkylating reagent tris(2-chloroethyl)amine (TCEA) was used as a trifunctional cross-linking reagent with a cross-linking span of 5 Å for myosin subfragment 1 (S-1). When S-1 was incubated with TCEA, all three domains of 20, 26, and 50 kDa in the S-1 heavy chain were cross-linked via the highly reactive sulfhydryl group SH_1 (Cys-707) on the 20-kDa domain. The cross-linking was accelerated by nucleotides. The present observation is consistent with the proposal that SH_1 is close to both the 26- and 50-kDa domains of S-1 and that movement within S-1 associated with the nucleotide binding occurs around SH_1 as well as around another reactive thiol, SH_2 (Cys-697) [Lu, R. C., Moo, L., & Wong, A. G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6392–6396; Hiratsuka, T. (1987) *Biochemistry 26*, 3168–3173].

There are three domains, the 20-, 26-, and 50-kDa domains, in the globular head region of the myosin molecule, i.e., myosin subfragment 1 (S-1).[1] S-1 contains the sites responsible for ATPase activity and binding of actin (Mueller & Perry, 1962). Two highly reactive sulfhydryl groups, SH_1 (Cys-707) and SH_2 (Cys-697) located on the 20-kDa domain (Balint et al., 1978), have been extensively studied since their modification was found to affect dramatically the ATPase activities of myosin (Sekine & Kielley, 1964). Furthermore, Lu et al. (1986) and Hiratsuka (1987a) demonstrated that SH_1 and SH_2 are close to both the 26- and 50-kDa domains, suggesting that all three domains of S-1 are contiguous around the SH_1–SH_2 region. Therefore, we hoped that an SH_1- or SH_2-directed trifunctional chemical reagent would be possible for cross-linking the three domains of S-1. If it were possible to cross-link the three domains, it would be possible to directly study the properties of a site where all three domains are contiguous. Such a study is very interesting for the reason that a direct communication between the ATP- and actin-binding sites may pass through the domain–domain contact region (Morales et al., 1982; Hiratsuka, 1984, 1985, 1986).

At the present time various types of alkylating reagents are used in the chemotherapy of neoplastic diseases (Calabresi & Parks, 1985). The reagents have in common the property of undergoing strongly electrophilic chemical reactions with the target molecules. These reactions result in the formation of covalent linkages (alkylation) with various nucleophilic substances, including such biologically important residues in proteins as sulfhydryl and amino groups. Among the chemotherapeutic alkylating reagents, nitrogen mustards TCEA and BCEA (Figure 1) are of particular interest as they seem to act as trifunctional and bifunctional reagents for proteins, respectively, with a cross-linking span of 5 Å. However, the use of TCEA and BCEA for the protein–protein cross-linking has not previously been reported.

In this paper we report the intramolecular cross-linking of S-1 with TCEA and BCEA. We found that three domains of S-1 are successfully cross-linked with the trifunctional reagent TCEA via SH_1 on the 20-kDa domain. Furthermore, the cross-linking was accelerated by Mg-ATP. We confirm previous reports that SH_1 is close to both the 26- and 50-kDa domains and that movement within S-1 associated with nucleotide binding occurs around the SH_1–SH_2 region (Lu et al., 1986; Hiratsuka, 1987a).

Materials and Methods

Reagents. TCEA, BCEA, and IAEDANS were purchased from Aldrich Chemical Co. α-Chymotrypsin, diphenylcarbamyl chloride treated trypsin, and soybean trypsin inhibitor were from Sigma Chemical Co. ATP, ADP, and AMP were from Kohjin Co. All other reagents were of reagent or biochemical research grade.

Preparations of Proteins. Rabbit skeletal myosin was prepared by the method of Perry (1955) with slight modification. S-1 was prepared by chymotryptic digestion of myosin as described by Weeds and Taylor (1975). Tryptic S-1 was prepared as described previously (Hiratsuka, 1986).

Specific Modification of Tryptic S-1. The specific labeling of SH_1 of tryptic S-1 with IAEDANS was done according to the procedure of Takashi (1979) as described previously (Hiratsuka, 1987a).

Cross-Linking Experiments. Tryptic and undigested S-1's (1.2–1.6 mg/mL) were incubated with BCEA or TCEA in 40 mM triethanolamine/NaOH (pH 8.4), 50 mM NaCl, and 0.5 mM $MgCl_2$ in the presence and absence of 0.5 mM ligand at 25 °C. BCEA and TCEA were dissolved in dimethylformamide immediately before use. Final concentrations of the reagent and dimethylformamide were 6 mM (BCEA) or 2 mM (TCEA) and 2%, respectively. The cross-linking reaction was stopped by the addition of 1 M glycine/NaOH (pH 8.0) or 2-mercaptoethanol when necessary at a final concentration of 0.67 or 0.14 M, respectively.

ATPase Measurements. The Ca^{2+}- and K^+-EDTA-ATPase activities were measured at 25 °C in 1 mM ATP, 0.5 M KCl, and 50 mM Tris-HCl (pH 8.0) in the presence of 5 mM $CaCl_2$ and EDTA, respectively. P_i liberated was determined by the method of Fiske and SubbaRow (1925).

Tryptic Digestion of S-1. Limited cleavage by trypsin was performed at 25 °C for 90 min in 40 mM triethanolamine/NaOH (pH 8.4), 50 mM NaCl, and 0.5 mM $MgCl_2$ at a

[1] Abbreviations: S-1, myosin subfragment 1; TCEA, tris(2-chloroethyl)amine; BCEA, methylbis(2-chloroethyl)amine; IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; SDS, sodium dodecyl sulfate; P_i, orthophosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton(s).

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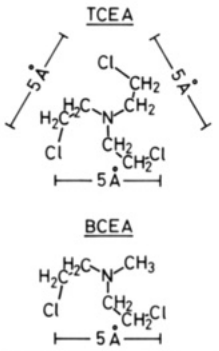

FIGURE 1: Structures of TCEA (upper) and BCEA (lower).

molar ratio of 1:100 for trypsin/S-1.

SDS–Polyacrylamide Gel Electrophoresis. Cross-linked proteins were analyzed by SDS–polyacrylamide gel electrophoresis by the system of Weber and Osborn (1969) in 7.5% acrylamide gels. To follow the process of cross-linking of tryptic S-1, 50–200-μL aliquots of sample were withdrawn periodically and pipetted into 3-fold volumes of a solution containing 0.1 M phosphate (pH 7.0), 2% SDS, 10% 2-mercaptoethanol, and 34% sucrose. Gels were stained with Coomassie Brilliant Blue.

Protein Determinations. The concentration of S-1 was determined from the extinction coefficient ($A^{1\%}_{1cm}$) at 280 nm of 7.5 (Wagner & Weeds, 1977). Protein concentrations of modified S-1 were determined by the biuret method (Gornall et al., 1949), standardized with the $A^{1\%}_{1cm}$ of unmodified S-1.

RESULTS

Cross-Linking of Tryptic S-1. Digestion with trypsin of S-1 produces three main peptide fragments corresponding to the 50-, 26-, and 20-kDa domains from the heavy chain (Balint et al., 1978; Mornet et al., 1979). It is well-known that although the tryptic digestion of S-1 lowers the protein's affinity for actin, it does not have a significant effect on the ATPase properties (Mornet et al., 1979; Botts et al., 1982; Hiratsuka, 1986). These properties of tryptic S-1 allow ready use of the protein in various experiments as a useful model for myosin ATPase. Thus, we first studied the reaction of TCEA and BCEA with tryptic S-1, which mainly consists of 26-, 50-, and 20-kDa peptide fragments.

First we examined the time course of the reaction of tryptic S-1 with the trifunctional reagent TCEA at a concentration of 2 mM and 25 °C. The experiment shown in Figure 2 (left) indicates that concomitantly with the formation of the 74-kDa cross-linked product there is a progressive decrease in the amounts of the original 20- and 50-kDa peptides. At prolonged reaction times (longer than 20 min), the 74-kDa product was converted into the 104-kDa product, accompanying a disappearance of the 26-kDa domain. After 120 min of reaction, most of the three domains was cross-linked, generating the major 104-kDa product and the minor 74-kDa product. When the denatured tryptic S-1 was incubated with TCEA, none of the cross-linked products was formed, suggesting that we are looking only at the existing interactions among three domains (not shown). It should be noted that TCEA scarcely forms any cross-link between the 20- and 26-kDa domains and between the 26- and 50-kDa domains. Although new protein bands of cross-linked products of 77 and 45 kDa were observed, their amounts were negligibly small (less than 5% of the 74- and 104-kDa products).

For comparative purposes we then tested the bifunctional reagent BCEA, which possesses the same functional group as TCEA (Figure 1), as a cross-linking reagent for tryptic S-1.

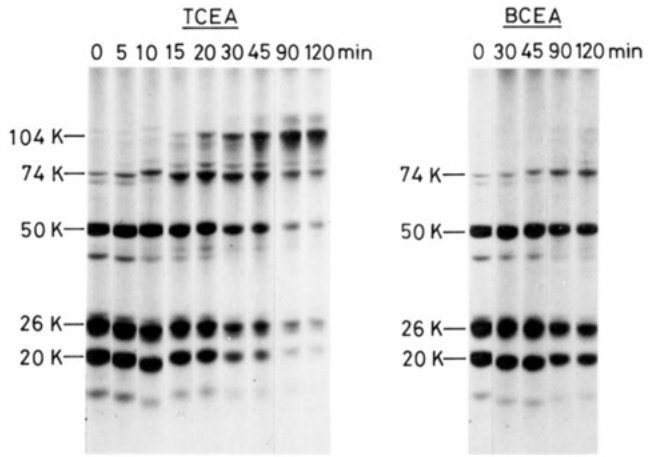

FIGURE 2: Time courses of the cross-linking reaction of tryptic S-1 with TCEA (left) and BCEA (right). The protein (1.6 mg/mL) was incubated with TCEA (2 mM) or BCEA (6 mM) in 40 mM triethanolamine/NaOH (pH 8.4), 50 mM NaCl, and 0.5 mM $MgCl_2$ at 25 °C. At the times indicated, samples were submitted to SDS–polyacrylamide gel (7.5%) electrophoresis.

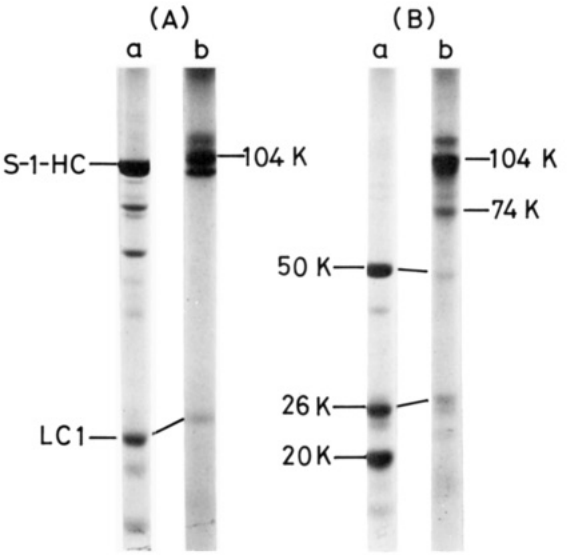

FIGURE 3: Cross-linking of intact S-1 with TCEA. S-1 (1.3 mg/mL) was incubated with 2 mM TCEA for 120 min. After addition of 2-mercaptoethanol (0.14 M), the reaction mixture was dialyzed against 40 mM triethanolamine/NaOH (pH 8.4), 50 mM NaCl, and 0.5 mM $MgCl_2$ and then subjected to tryptic cleavage for 90 min with a molar ratio of 1:100 for trypsin/S-1. (A) Before tryptic cleavage; (B) after tryptic cleavage. (a) Control S-1; (b) cross-linked S-1. HC, heavy chain; LC1, light chain 1.

In this case, no cross-linked product of 104 kDa was observed even at a molar quantity of BCEA triple that of TCEA (Figure 2, right). Only the 74-kDa product was generated even after 120 min of reaction.

Cross-Linking of Intact S-1. In a second phase of this work, we investigated the cross-linking of intact S-1 with TCEA. S-1 was incubated for 120 min with 2 mM TCEA and examined by SDS–polyacrylamide gel electrophoresis. As shown in Figure 3A, the majority of the heavy chain of cross-linked S-1 had lower mobility than unlabeled heavy chains. Upon trypsin cleavage, a new 74-kDa band was generated while the original 95-kDa band disappeared but the original 104-kDa band remained unchanged (Figure 3B). This electrophoretic pattern was essentially the same as that of tryptic S-1 which was incubated with TCEA for 120 min (Figure 2). On the other hand, heavy chains of control S-1 were thoroughly converted into the 20-, 26-, and 50-kDa peptide fragments under this

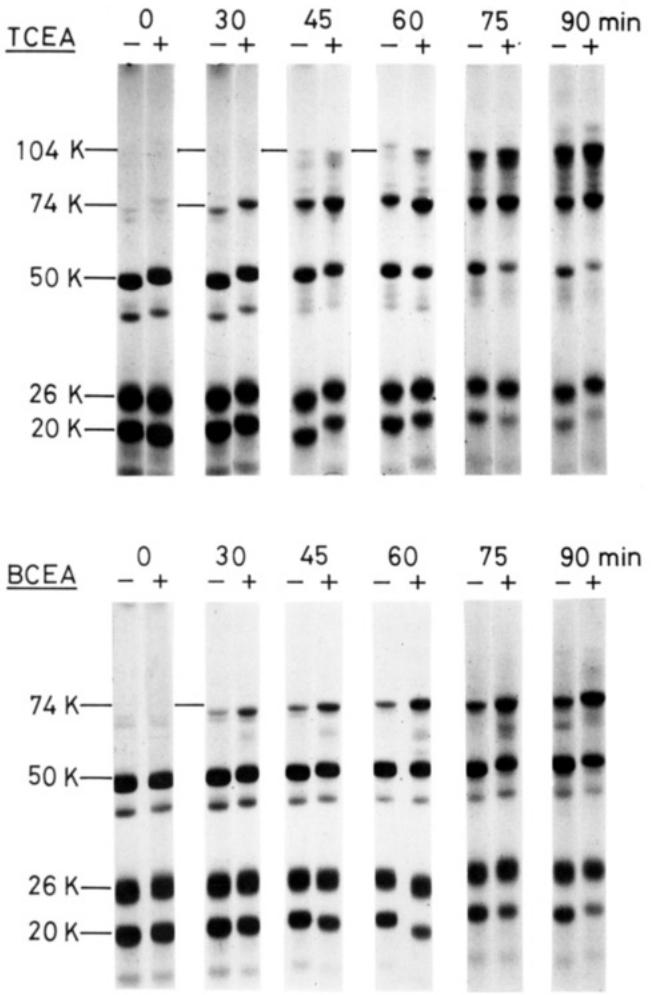

FIGURE 4: Cross-linking of tryptic S-1 with TCEA (upper) and BCEA (lower) in the presence of ATP. The protein (1.2 mg/mL) was incubated with TCEA (2 mM) or BCEA (6 mM) in the presence (+) and absence (−) of 0.5 mM ATP. At the times indicated, samples were submitted to SDS–polyacrylamide gel (7.5%) electrophoresis.

condition. These results suggest that three domains in intact S-1 are also cross-linked with TCEA in a manner similar to that in the case of tryptic S-1.

Effects of Nucleotides on the Cross-Linking Reaction. We also examined whether ATP changes the formation of the cross-linked products. As shown in Figure 4 (upper), the addition of 0.5 mM ATP to the incubation mixture of TCEA and tryptic S-1 accelerated the formation of not only the 74-kDa product but also the 104-kDa product. The nucleotide effect was also observed in the case of the reaction with BCEA (Figure 4, lower). The formation of the 74-kDa cross-linked product was accelerated by the addition of ATP. Again, no cross-linked product of 104 kDa was generated even in the presence of ATP. Effects of nucleotides other than ATP and P_i on the formation of the 74-kDa cross-linked product were examined with BCEA (Figure 5). Like ATP, ADP accelerated the formation of the product. However, no effect was observed with AMP and P_i, which are neither substrate nor competitive inhibitor for the myosin S-1 ATPase. Similar results were obtained in the case of the cross-linking with TCEA (not shown).

Determination of Cross-Linking Sites. To obtain information about the cross-linking sites of three domains of S-1, we first measured the ATPase activities after cross-linking of S-1. Figure 6 shows the time course of the changes in ATPase activities when S-1 was incubated with TCEA (upper) and BCEA (lower). In both cases, the K^+-EDTA-ATPase activity

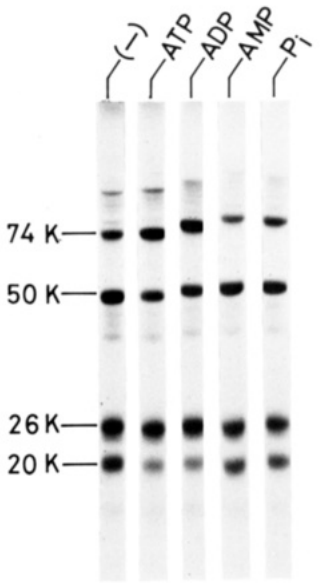

FIGURE 5: Effects of adenine nucleotides and P_i on the formation of cross-linked products of tryptic S-1. Tryptic S-1 was incubated with 6 mM BCEA for 90 min in the presence and absence of 0.5 mM ligand; (−) minus ligand.

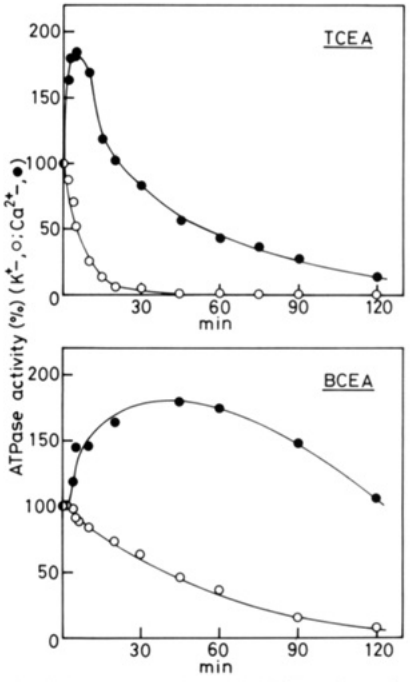

FIGURE 6: Effect of the reaction with TCEA (upper) and BCEA (lower) on the Ca^{2+}- (●) and K^+-EDTA- (○) ATPase activities of S-1. S-1 was incubated with TCEA or BCEA as described in Figure 2. At the times indicated, the cross-linking reaction was stopped by the addition of 0.67 M glycine/NaOH (pH 8.0) and subjected to ATPase assays. The Ca^{2+}- and K^+-EDTA-ATPase activities were measured at 25 °C in 1 mM ATP, 0.5 M KCl, and 50 mM Tris-HCl (pH 8.0) in the presence of 5 mM $CaCl_2$ and EDTA, respectively.

became inhibited, while the Ca^{2+}-ATPase activity was initially elevated up to 180% the control value, but on subsequent standing it was progressively lost. We interpret such a change in the ATPase activities as indicating that the highly reactive sulfhydryl group SH_1 is initially blocked at a rapid rate and then another reactive sulfhydryl group, SH_2, is blocked at a lower rate (Sekine & Kielley, 1964; Yamaguchi & Sekine, 1966).

Although both SH_1 and SH_2 are located on the 20-kDa domain (Balint et al., 1978), comparison of the time course of the change in ATPase activities (Figure 6) with the elec-

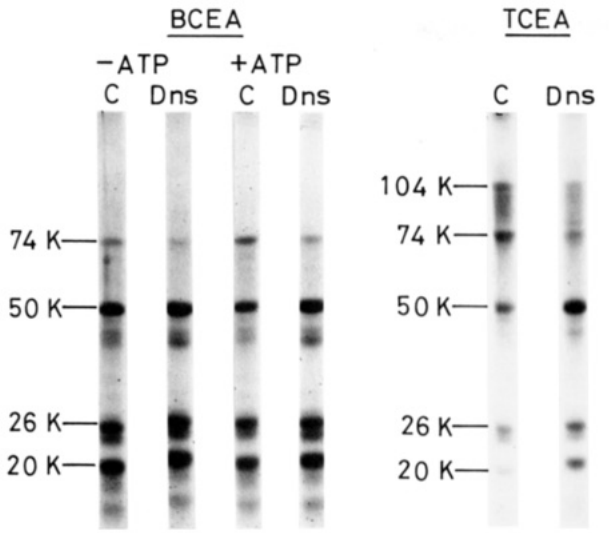

FIGURE 7: Effect of blocking of SH_1 of tryptic S-1 on the cross-linking reaction with BCEA (left) and TCEA (right). C, control tryptic S-1; Dns, SH_1 of tryptic S-1 was blocked with IAEDANS (Takashi, 1979). The protein was incubated with BCEA (6 mM) or TCEA (2 mM) for 60 min in the presence and absence of 0.5 mM ATP.

trophoretic pattern of cross-linking (Figure 2) suggests that SH_1 instead of SH_2 is involved in the cross-linking of the 20- and 50-kDa domains in both cases of TCEA and BCEA. In order to clarify this point further, we next prepared the tryptic S-1 derivative in which SH_1 was blocked by the typical SH_1 modifier IAEDANS (Takashi, 1979). When SH_1 of tryptic S-1 was preblocked with IAEDANS, the protein scarcely generated the 74-kDa cross-linked product even after a 60-min incubation with BCEA, suggesting the involvement of SH_1 in the cross-linking (Figure 7, left). This abolition of the product formation was also observed in the presence of ATP. A similar result was obtained in the case of TCEA cross-linking; both the 74- and 104-kDa products were scarcely formed (Figure 7, right). Traces of the 74- and 104-kDa products seen in the SH_1-blocked sample were taken as those from the unlabeled protein, since thiol titration indicated 0.1–0.2 mol of SH_1/mol of the protein remained unlabeled.

In conclusion, BCEA and TCEA first react with SH_1 on the 20-kDa domain and then with a residue on the 50-kDa domain, generating the 74-kDa product. In the case of the trifunctional reagent TCEA, the 26-kDa domain is finally cross-linked to the 74-kDa product.

Discussion

Our present results show that both TCEA and BCEA first cross-link the 20- and 50-kDa domains via SH_1 instead of SH_2. Both reagents have the property of undergoing strongly electrophilic chemical reaction especially with sulfhydryl and amino groups in proteins (Calabresi & Parks, 1985). The reactivity of both reagents is based on the presence of the 2-chloroethyl group (Figure 1). It is well-known that such alkyl halides specifically label the highly reactive SH_1 of S-1: monobromobimane and dibromobimane (Mornet et al., 1985; Ue, 1987) and 4-(bromoethyl)-6,7-dimethoxycoumarin (Hiratsuka, 1987b). Thus, it is reasonable to conclude that TCEA and BCEA first react with SH_1 of S-1 and then with a residue on the 50-kDa domain, generating the 74-kDa product.

In the case of TCEA, the 74-kDa product was further cross-linked to the 26-kDa domain, generating the 104-kDa product. Our ATPase measurement suggests that SH_2 in the 20-kDa domain is modified with TCEA. However, the involvement of SH_2 in the cross-linking of the 26-kDa domain is unlikely, since BCEA, which possesses the same functional group (the 2-chloroethyl group) and the same cross-linking span as TCEA (Figure 1), failed to cross-link the 26-kDa domain to the 74-kDa product. Neither raising the BCEA concentration nor the addition of nucleotides, which are known to increase considerably the reactivity of SH_2 (Yamaguchi & Sekine, 1966), resulted in the formation of the 104-kDa cross-linked product [Figure 2, Figure 4 (lower), and Figure 5]. Thus, the involvement of SH_2 in the cross-linking of the 26-kDa domain is unlikely. Taking into account the fact that both TCEA and BCEA decrease the Ca^{2+}-ATPase activity (Figure 6), SH_2 seems to be monofunctionally modified with both reagents, generating no cross-linked product.

It is interesting that the trifunctional reagent TCEA cross-links successfully three domains of S-1 but the bifunctional reagent BCEA does not although both reagents possess the same functional group and the same cross-linking span (Figure 1). Taking into account the result that the cross-linking of the 26-kDa domain occurs only after the 20- and 50-kDa domains are cross-linked (Figure 2), the reason appears to be as follows. In the case of BCEA the functional group attached to the 26-kDa domain has no partner for cross-linking on the 20- and 50-kDa domains since the cross-linking sites on the latter domains are occupied by another BCEA molecule. In contrast to this, one TCEA molecule can afford a cross-link for the 26-kDa domain even after its two functional groups are used as a cross-link between the 20- and 50-kDa domains. It is unlikely that after the 20- and 50-kDa domains are cross-linked another TCEA molecule acts as a *bifunctional* reagent to cross-link the 26-kDa domain to the 74-kDa product since the bifunctional reagent BCEA can never generate the 104-kDa product. Thus, it is more likely that at first two chloroethyl groups of TCEA are used as a cross-link between SH_1 and the 50-kDa domain and then the residual functional group is used as a cross-link between the 26-kDa domain and the 74-kDa product. This is consistent with the result that three domains of S-1 are contiguous around SH_1 (Lu et al., 1986).

Upon addition of Mg-ATP to the incubation mixture of TCEA and tryptic S-1, the formation not only of the 74-kDa product but also of the 104-kDa product was accelerated. The reason that the nucleotide enhances the reactivity of SH_1 against the cross-linking reagent is ruled out, since Mg-ATP protects SH_1 from the alkylating reagent *N*-ethylmaleimide (Schaub et al., 1975). Our results favor the interpretation that binding of the nucleotide induces conformational changes which cause the 50-kDa domain to become close to SH_1 on the 20-kDa domain and the 26-kDa domain to become close to the 74-kDa cross-linked product, resulting in the acceleration of the formation of the 74- and 104-kDa cross-linked products.

The above interpretation is consistent with recent suggestions that a highly flexible primary loop exists around SH_1 (Mornet et al., 1985) and that the ATPase site of S-1 resides at or near the region where the 20-, 26-, and 50-kDa domains are contiguous (Hiratsuka, 1987a). The proximity of the ATPase site to such a specific region would be favorable to intersite communication between the ATPase and actin-binding sites. This paper has led to speculations that peptide chain distortions generated by ATP bound at the ATPase site would be transmitted to the actin-binding site through the domain–domain contact regions (Hiratsuka, 1984).

In conclusion, nitrogen mustards BCEA and TCEA with a cross-linking span of 5 Å are useful as sulfhydryl and amino group specific cross-linking reagents for proteins. Especially,

the trifunctional reagent TCEA provides a new tool for studying protein-protein contacts in various proteins which possess multisubunit and multidomain structures. Its utility best can be appreciated in this paper by the success in the cross-linking of three domains of myosin S-1.

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Registry No. ATPase, 9000-83-3; TCEA, 555-77-1; BCEA, 51-75-2.

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Origin of Viscosity Effects in Carbonic Anhydrase Catalysis. Kinetic Studies with Bulky Buffers at Limiting Concentrations†

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ABSTRACT: In our earlier paper we showed that the rates of CO_2 hydration and HCO_3^- dehydration catalyzed by the high-activity form of mammalian erythrocyte carbonic anhydrase (CA II) were dependent on solution viscosity increase and that the effect was linked to some kind of proton-transfer-related event [Pocker, Y., & Janjič, N. (1987) *Biochemistry 26*, 2597-2606]. In order to further elucidate the source of the observed viscosity effect, the dependence of k_{cat} and K_m for CA II catalyzed HCO_3^- dehydration at pH 5.90 on sucrose-induced viscosity increase was investigated at several concentrations of 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, including the very low buffer concentration region (<10 mM) where the proton transfer between the shuttle group on the enzyme and buffer becomes rate limiting. In all examined cases, k_{cat} steadily decreased with added sucrose while K_m remained independent of the viscosity increase. The extent to which this reaction was dependent on viscosity was found to be constant, within experimental error, over the entire range of MES buffer concentrations studied (1-20 mM). Furthermore, the viscosity effect was qualitatively and quantitatively the same when an exceptionally large buffer (i.e., bovine serum albumin) was used instead of the more commonly used biological buffer (i.e., MES). We conclude from these observations that it is unlikely that reduction in translational diffusion rates of the substrate or the buffer species contributes significantly to the observed viscosity effect and present evidence that supports our assertion that the rate-limiting proton transfer between the zinc-water or zinc-hydroxide and buffer is affected through viscosity- (or cosolute-) induced changes in intramolecular isomerization rates involving functionally important motions.

The only known physiological role of carbonic anhydrase is in catalyzing the interconversion of CO_2 and HCO_3^- (eq 1).

$$EH^+ + HCO_3^- \rightleftharpoons E + CO_2 + H_2O \quad (1)$$

With a turnover number close to 1×10^6 s^{-1} at 25 °C, the high-activity form of mammalian erythrocyte carbonic anhydrase (CA II)[1] is one of the fastest enzymes known (Edsall, 1967; Khalifah, 1971; Pocker & Sarkanen, 1978; Lindskog,

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[1] Abbreviations: CA II, carbonic anhydrase, high-activity isozyme; MES, 2-(*N*-morpholino)ethanesulfonic acid; BSA, bovine serum albumin; BCP, bromocresol purple.

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