The Effect of a Cross-Bridging Thiol Reagent on the Catecholamine Fluxes of Adrenal Medulla Vesicles

By W. HASSELBACH AND G. TAUGNER

Max-Planck-Institut für medizinische Forschung, Abteilung Physiologie, 69 Heidelberg, Germany

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The thiol groups of the vesicular protein of bovine adrenal medulla were allowed to react with the bifunctional thiol reagent bis-(*N*-maleimidomethyl) ether and with the monofunctional thiol reagent *N*-ethylmaleimide, and the ATP-dependent and -independent catecholamine fluxes of the modified preparations were studied. 1. During the initial phase of the reaction bis-(*N*-maleimidomethyl) ether blocks twice as many thiol groups as does *N*-ethylmaleimide at equimolar concentrations. 2. Labelling of the bis-(*N*-maleimidomethyl) ether-protein compound with [¹⁴C]-cysteine shows that 70–80% of the blocked thiol groups are interconnected by the bifunctional thiol reagent. 3. At a low extent of reaction (1.5 mol of thiol groups/10⁶ g of protein) the catecholamine efflux is diminished. If more than 2 mol of thiol groups/10⁶ g of protein are blocked, the efflux is enhanced whichever thiol reagent is applied. 4. If 2–4 mol of thiol groups/10⁶ g of protein are blocked the inhibition of the catecholamine influx increases linearly with the proportion of the thiol groups blocked. 5. ATP protects the catecholamine influx and the adenosine triphosphatase activity against bis-(*N*-maleimidomethyl) ether poisoning somewhat less effectively than against *N*-ethylmaleimide poisoning.

The reaction of 4–5 mol of thiol groups/10⁶ g of protein of the catecholamine vesicles with the monofunctional thiol reagent NEM* blocks the ATP-dependent catecholamine influx and greatly increases the catecholamine efflux (Taugner & Hasselbach, 1968). ATP protects the vesicles from these effects of NEM, although it diminishes the extent of reaction of thiol groups by only 10%. Obviously the functionally relevant thiol groups comprise only a small percentage of the total thiol groups, most of which can be blocked without affecting the function (Taugner & Hasselbach, 1968).

These studies have now been extended by using bifunctional thiol reagents for reaction with the vesicular thiol groups. The use of specific thiol reagents other than NEM should furnish further evidence for the involvement of thiol groups in catecholamine transport and adenosine triphosphatase activity. Further, bifunctional reagents can usefully be applied for the detection of structural changes in the vesicular proteins involved in catecholamine transport. In most schemes describing the translocation of charged or uncharged particles through biological membranes, the occur-

rence of structural or conformational changes in the membranes has been considered (cf. Hasselbach & Seraydarian, 1966; Jardetzky, 1966; Sen & Post, 1965; Skou, 1965; Whittam & Ager, 1964). If during passage through the membrane the relative position of neighbouring thiol groups changes, the linkage of these groups by a bifunctional thiol reagent should interfere with the transport processes. These groups may not be identical with and may be localized apart from those groups whose blockage by monofunctional reagents produces inactivation.

For haemoglobin Simon & Konigsberg (1966) were able to provide evidence for such effects with the bifunctional thiol reagent BME. It presumably connects the cysteine residue β -93 with an unknown residue in the β -chain. This kind of substitution prevents the Bohr effect. Presumably the cross-bridge formed by the reagents prevents the conformational change causally linked with the Bohr effect.

MATERIALS AND METHODS

The catecholamine-storage vesicles of bovine adrenal medullas were isolated in unbuffered 0.3M-sucrose at 2–4°C by using the method described by Taugner & Hasselbach (1966). The adrenal medullas of six to eight

* Abbreviations: NEM, *N*-ethylmaleimide; BME, bis-(*N*-maleimidomethyl) ether; PDM, 1,2-dimaleimidobenzene.

animals were passed through a nylon sieve and homogenized; nuclei and unbroken particles were removed by centrifugation at 900g for 6min and the supernatant was centrifuged at 12000g for 15min. The storage vesicles of higher density were separated from the upper, loosely packed layer of vesicles with a lower catecholamine/protein ratio. The latter was discarded by swirling with ice-cold 0.3M-sucrose. This procedure was repeated twice after centrifugation at 9000g and 7000g respectively. The catecholamine/protein ratio had a final value of about 2.75 (2.12–3.20) $\mu\text{mol/mg}$ of protein (mol.wt. of catecholamine 180). A sample was taken from each preparation for determination of the protein content by the Kjeldahl method. The catecholamine content was measured colorimetrically by the method of von Euler & Hamberg (1949).

Reaction of thiol groups. Isolated catecholamine-storage vesicles were incubated in 0.3M-sucrose buffered with sodium glycerophosphate to pH 7.4 in the presence of NEM or BME at 31°C with constant stirring. The assay mixtures contained 1.5–2.9mg of protein/ml. At time-intervals from 30s to 30min portions (0.5ml) were taken and transferred into reaction vessels containing 2ml of 0.5mM- ^{14}C NEM in 10% (v/v) ethanol buffered with sodium glycerophosphate to pH 7.4 to label the thiol groups of the vesicular protein that remained free after having been treated with the non-radioactive thiol reagents. The difference between the ^{14}C NEM content of untreated preparations and that treated with unlabelled NEM or BME represents the number of mol of thiol groups/ 10^6 g of protein blocked by the unlabelled thiol reagent. After 9–12h in contact with ^{14}C NEM the protein was precipitated with 10% (w/v) trichloroacetic acid, washed twice with 2.4% (w/v) trichloroacetic acid in 60% (v/v) methanol, dissolved in Hyamine and transferred into the scintillation mixture for measurement of radioactivity.

^{14}C Cysteine-labelling of the protein-bound BME or PDM was measured to decide whether the bifunctional thiol reagents had reacted with one or both of their reactive groups. Other samples (0.5ml) were taken at the same time as those for the determination of thiol groups. They were transferred into reaction vessels containing 1ml of 1mM- ^{14}C cysteine in buffered 0.3M-sucrose (pH 7.4) and allowed to react for 15min with constant shaking. Then 0.2ml of 2-mercaptoethanol was added. After 10min the protein was precipitated with 5ml of 10% (w/v) trichloroacetic acid. The precipitate was washed four times with 6% (w/v) trichloroacetic acid and transferred with Hyamine into the scintillation mixture.

Measurement of catecholamine fluxes. Catecholamine fluxes across the vesicular membrane of preparations treated with NEM or BME were measured by using ^{14}C adrenaline. Incubation was carried out at 31°C with constant stirring in a solution containing (final concentrations): approx. 0.75mg of protein/ml (0.45–1.0mg/ml), ATP + Mg^{2+} (5mM) and sodium glycerophosphate buffer, pH 7.4 (0.04M), in 0.3M-sucrose. The thiol reagents were added to the incubation mixture either 5min before or after the addition of ATP. At chosen time-intervals after the addition of ^{14}C adrenaline 2ml samples were taken, cooled for 5min in ice-water and centrifuged at 15000g for 15min. The vesicular sediment was extracted with

0.5ml of 10% (w/v) trichloroacetic acid overnight and transferred into the scintillation mixture. The initial velocity of the catecholamine influx was calculated from the average of three values of the first 10min of the experiment according to the expression:

$$v_0 = (a_0/t_1) \ln(a_1/a_0)$$

where t_1 is the time (min) after the start of the experiment, a_0 is the radioactivity (c.p.m.) in the medium at zero time, a_1 is the radioactivity (c.p.m.) in the medium at time t_1 and v_0 represents the rate of decrease of the radioactivity (c.p.m.) in the medium. The catecholamine flux is obtained by multiplying v_0 with the specific radioactivity of adrenaline. The specific radioactivity of the added ^{14}C adrenaline was 38mCi/mmol. The specific radioactivity in the media, however, was found to be lower, because catecholamine is released from the vesicles. The rate of the release of the intravesicular catecholamines was determined by measuring the increasing catecholamine content in the medium during the initial 10min of the experiment.

Measurement of radioactivity. The ^{14}C radioactivity was measured in a Packard Tri-Carb model 4000 spectrometer in a scintillation mixture consisting of toluene-Triton X-100 (11:4, v/v) containing 4g of Omnifluor [98% 2,5-diphenyloxazole + 2% *p*-bis-(*o*-methylstyryl)-benzene]/l.

Assay of adenosine triphosphatase activity. The adenosine triphosphatase activity was measured at 31°C in the same medium as described above. The thiol reagents were added to the preparation either 5min before or 5min after the addition of ATP. The reaction was stopped after chosen time-intervals by treatment of samples with 10% (w/v) trichloroacetic acid. The rates of hydrolysis of ATP were calculated from two successive 5min intervals after the preparation had been in contact with the thiol reagent for 5min. The P_i liberated was determined by the method of Rockstein & Herron (1951).

Chemicals. BME was synthesized by the method described by Tawney *et al.* (1961). 1,2-Dimaleimido-benzene was from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A., NEM was from EGA Chemie K.G. Keppler u. Reif, Steinheim/Albuch, Germany, ^{14}C NEM was from Schwarz Bioresearch Inc., Orangeburg, N.Y., U.S.A., DL- ^{14}C adrenaline was from Farbwerke Hoechst, Frankfurt am Main, Germany, ^{14}C cysteine was from The Radiochemical Centre, Amersham, Bucks., U.K., ATP (disodium salt) was from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A., and Omnifluor was from NEN Chemicals G.m.b.H., Frankfurt am Main, Germany.

RESULTS

Reaction of the membranal thiol groups by BME and NEM. Fig. 1(a) shows that the initial rate at which the vesicular thiol groups react with NEM or BME increases only twofold when the concentrations of the reagents are raised from 0.01mM to 0.1mM. Kinetics like these suggest that the substitution is preceded by the formation of a high-affinity adsorption complex between the protein and the reagents (Green, 1963). The rates as well

Fig. 1. Time-course of thiol substitution of the vesicular protein by equimolar (a) and equivalent (b) concentrations of mono- and bi-functional thiol reagents. (a) Δ , NEM (0.01 mM); $\circ$, BME (0.01 mM); $\blacktriangle$, NEM (0.1 mM); $\bullet$, BME (0.1 mM); $\square$, 1,2-dimaleimidobenzene (0.01 mM); $\blacksquare$, 1,2-dimaleimidobenzene (0.1 mM); (b) Δ , NEM (0.02 mM); $\circ$, BME (0.01 mM); $\blacktriangle$, NEM (0.2 mM); $\bullet$, BME (0.1 mM). The reaction was carried out under standard conditions as given in the Materials and Methods section. The number of mol of thiol groups/10⁶ g of protein blocked by the thiol reagents was determined indirectly: the thiol groups that remained free after treatment with the non-radioactive thiol reagents were labelled with [¹⁴C]NEM, and the number found subtracted from the total of thiol groups that react with [¹⁴C]NEM during an incubation period of 9–12 h.

as the extents of reaction reached after 30 min are nearly identical if both reagents are applied in equivalent concentrations, those with 0.1 mM-BME corresponding to those with 0.2 mM-NEM (Fig. 1b). This behaviour indicates that most of the bifunctional molecules react with two thiol groups each.

Although the reagents were applied under all conditions in excess, no more than 4.5 mol of thiol groups/10⁶ g of protein are blocked in the native protein. These represent the more readily accessible groups from a total of approx. 10 thiol groups/10⁶ g of protein reacting after a prolonged incubation.

When reaction with [¹⁴C]NEM is carried out in the presence of ATP the extent of reaction is 1 mol of thiol groups/10⁶ g of protein less than in the absence of ATP. The lack of radioactively labelled BME and technical difficulties made it impossible to test whether ATP also protects the membranal thiol groups against reaction with BME. This was because ATP would interfere with the labelling by [¹⁴C]NEM of the residual thiol groups left unsubstituted by BME, and it proved impossible completely to remove the ATP without loss of protein.

Cross-bridge formation between adjacent thiol groups. Vesicles that had been treated with a bifunctional reagent were incubated with excess of [¹⁴C]cysteine (1 mM) to discover how much of the attached reagent had been bound by only one of its functional groups. The percentage (α) of the bifunctional molecules anchored by only one of their reactive groups is given by:

$$\alpha = \frac{100c}{(b-c)/2 + c}$$

where b is the number of thiol groups blocked by the bifunctional reagent and c is the number of cysteine groups subsequently bound.

When 0.01 mM-BME has been allowed to react with the protein (3 mg/ml) for 5 min a value for α of only 20% is found. This means that more than 80% of the BME molecules are attached with both functional groups. When higher concentrations of the bifunctional reagents are applied the probability that neighbouring thiol groups of the membranal protein are blocked monofunctionally by different molecules of the reagent becomes higher. Consequently, a greater number of reactive groups remains free and can subsequently combine with cysteine; this was found to be 30% if 0.1 mM-BME was applied. As expected, monofunctional attachment occurs more often when 1,2-dimaleimidobenzene is used as bifunctional reagent because the distance between the reactive residues in this molecule is only approx. 5 Å (Fig. 2). The free maleimide groups of bound BME or 1,2-dimaleimidobenzene disappear slowly in a secondary reaction. This decline occurs with the same rate irrespective of the concentration of the reagent used.

Catecholamine influx and efflux. In sucrose-containing solutions at 30°C the catecholamine concentration of isolated vesicles of the adrenal medulla declines at a rate of 4–5 nmol/min per mg of protein if the suspension does not contain ATP. However, when ATP is present no catecholamine is released because the efflux of catecholamine is compensated for by an ATP-dependent influx (Taugner & Hasselbach, 1966). Both influx and efflux are affected if the membranal thiol groups of

the vesicles are blocked by NEM as well as by BME. The results are shown in Fig. 3 and Table 1.

The blockage of 1.5 mol of thiol groups/10⁶ g of protein, as occurs when the vesicles are incubated with 0.01 mM-NEM, produces a drastic decrease of the catecholamine influx from 4 to 1.3 nmol/min per mg of protein. When more thiol groups are blocked by NEM or by BME a further linear decline of the catecholamine influx is observed. Fig. 4 shows that the decline does not obviously depend on the type of reagent used but only on the number of thiol groups blocked.

Fig. 2. Decline with time of the fraction of molecules of bound bifunctional reagent attached by only one group. Δ, BME (0.01 mM); ▲, 1,2-dimaleimidobenzene (0.01 mM); ○, BME (0.1 mM); ●, 1,2-dimaleimidobenzene (0.1 mM). After the protein had reacted with the bifunctional thiol reagents for different time-intervals [¹⁴C]cysteine was added in excess (1 mM) to label the functional groups of the reagents that remained free. The numbers of monofunctionally bound bifunctional thiol reagent molecules were related to the total number of thiol groups blocked by the expression given in the Results section.

Fig. 3. Time-course of the catecholamine influx of NEM-treated and BME-treated catecholamine vesicles. ●, ATP+NEM (0.1 mM); Δ, ATP+BME (0.01 mM); ▲, ATP+BME (0.1 mM); ▽, BME (0.01 mM)+ATP. In the experiments marked ATP+NEM or BME, ATP was added 5 min before the thiol reagent; in those marked NEM or BME+ATP, ATP was added 5 min after the thiol reagent. The experiments were carried out as described in the Materials and Methods section and were started with the addition of [¹⁴C]-adrenaline. The catecholamine influx was calculated from the decline of the [¹⁴C]adrenaline radioactivity and the analytically determined catecholamine content in the medium.

Table 1. Catecholamine fluxes in preparations poisoned with NEM or BME

ATP was added either 5 min before (ATP+reagent) or 5 min after (reagent+ATP) the addition of the indicated thiol reagent. The experiments were started with the addition of [¹⁴C]adrenaline. The results are shown as means ± S.E.M. of the numbers of experiments given in parentheses.

Experimental conditions	Concn. of thiol reagent (mM)	Catecholamine influx, release and efflux (nmol/min per mg of protein)		
		Influx	Release	Efflux (release+influx)
ATP+NEM	0.01	4.18 ± 0.70 (4)	0.10 ± 0.08 (4)	4.28
NEM+ATP	0.01	1.27 ± 0.13 (4)	1.91 ± 0.52 (4)	3.18
ATP+NEM	0.1	3.99 ± 0.85 (4)	2.15 ± 0.63 (4)	6.14
NEM+ATP	0.1	0.67 ± 0.16 (4)	7.88 ± 0.78 (4)	8.55
ATP+BME	0.01	3.27 ± 0.19 (6)	2.58 ± 0.76 (6)	5.85
BME+ATP	0.01	0.82 ± 0.20 (6)	5.64 ± 0.92 (6)	6.46
ATP+BME	0.1	1.65 ± 0.10 (7)	7.24 ± 1.63 (7)	8.89
BME+ATP	0.1	0.36 ± 0.04 (7)	8.65 ± 1.72 (7)	9.01
ATP+NEM	0.05	2.81 (1)	2.20 (1)	5.01
ATP+BME	0.025	1.28 (1)	4.48 (1)	5.76

The efflux of catecholamine changes in a more complex manner when the extent of reaction of the thiol groups increases. The efflux rate is decreased at a low extent of reaction but rises steeply when more than 2 mol of thiol groups/10⁶ g of protein are blocked (Fig. 4). Again there is no obvious difference between blockage with NEM and BME.

The steep decline in the catecholamine influx produced by the reaction of the membranal thiol groups does not occur if the reaction is performed in the presence of ATP. The results given in Table 1 reveal that ATP counteracts the effect of reaction with BME less than it does that with NEM. Although ATP effectively protects catecholamine influx from the effects of NEM and BME, it has no

effect whatever on the enhancement of efflux evoked by either reagent. Consequently the vesicles always lose catecholamine when the membranal thiol groups are blocked.

Vesicular adenosine triphosphatase. When the thiol groups of the vesicles have reacted with BME or NEM the vesicular adenosine triphosphatase activity is partially inhibited. The degree of inhibition is related to the extent of reaction of the thiol groups (Table 2). As with the ATP-dependent catecholamine influx, the adenosine triphosphatase activity is also protected by ATP against the inhibiting effect of both reagents. Table 2 shows that the ATP-protected activity varies between 2 and 4.7 nmol/min per mg of protein, depending on the type of reagent and concentrations used. These values correspond quite well to the catecholamine influx rate determined under the same conditions. The ratio of catecholamine influx to the ATP-protected adenosine triphosphatase activity is approx. 0.7.

DISCUSSION

The blockage of the thiol groups of catecholamine vesicles of the adrenal gland interferes with the vesicular adenosine triphosphatase, the ATP-dependent catecholamine influx and the release of the catecholamines. There is no essential difference between the effects produced by mono- and bi-functional thiol reagents. All the observed changes must be attributed to the reactions of the thiol reagents with the membranal protein of the vesicles and not with their water-soluble protein fraction. Smith & Winkler (1967) have shown that the latter, which had been claimed to be essential for catecholamine storage and release, is characterized by a relatively low cysteine content (4 mol of cysteine residues/10⁶ g of protein). Only a small

Fig. 4. Relation between the catecholamine fluxes and the number of thiol groups blocked. The thiol reagents were added to the vesicular suspension 5 min before ATP. ■, Control; ○, NEM (0.01 mM); ●, NEM (0.1 mM); △, BME (0.01 mM); ▲, BME (0.1 mM). The catecholamine fluxes and the number of mol of thiol groups/10⁶ g of protein blocked (determined as given in Fig. 1) were obtained from separate experiments. The catecholamine fluxes were calculated as described in the Materials and Methods section.

Table 2. *Inhibition of the adenosine triphosphatase by thiol reagents*

The thiol reagents were added either 5 min before (reagent+ATP) or 5 min after (ATP+reagent) the addition of ATP. The results are shown as means $\pm$ s.e.m. of the number of experiments given in parentheses. Transport ratio =

$$\frac{\text{catecholamine influx (nmol/min per mg of protein)}}{B - A}$$

A and *B* are as defined in the table.

		Adenosine triphosphatase sp. activity (nmol of P _i /min per mg of protein)			
Thiol reagent	Control	Thiol reagent + ATP (A)	ATP + thiol reagent (B)	Difference (B - A)	Transport ratio
BME (0.01 mM)	25.1	12.9 $\pm$ 1.32 (9)	17.6 $\pm$ 1.14 (9)	4.7	0.7
BME (0.1 mM)		4.8 $\pm$ 0.43 (5)	7.3 $\pm$ 0.65 (4)	2.5	0.65
NEM* (0.1 mM)		6.9 $\pm$ 1.17 (7)	11.5 $\pm$ 1.09 (7)	4.6	0.87

* Results obtained by Taugner & Hasselbach (1968).

fraction of these residues react with NEM. The low incorporation of [^{14}C]NEM into the water-soluble protein fraction in comparison with the much higher degree of labelling of the membranal protein (G. Taugner & W. Hasselbach, unpublished work) is an additional proof of the specificity of the maleimide compounds as thiol reagents. Since the membranal protein comprises only 25–30% of the total protein, the blockage of 3–4 mol of thiol groups/ 10^6 g of protein in the total vesicular protein usually required for complete inactivation corresponds to 16–20 mol of thiol groups/ 10^6 g of membranal protein. This represents about 40–50% of all thiol groups of the membranal protein that can be blocked by NEM after a prolonged treatment.

The high specificity of the reaction with NEM, measured with [^{14}C]NEM, guarantees an unambiguous determination of the number of mol of blocked thiol groups/ 10^6 g of protein. On the other hand, the extent of reaction with BME had to be measured indirectly since radioactive BME was not available. The determined value includes thiol groups interconnected by BME and those connected with BME molecules of which one reactive group has remained free. From the latter fraction, which was measured by labelling with radioactive cysteine, and the total value for the thiol groups blocked by BME, the proportion of the thiol groups interconnected by BME was calculated. The finding that more than 60% of the thiol groups reacting with NEM can be interconnected by bifunctional reagents was unexpected because of the relatively low thiol content of the vesicular membranes. If the thiol groups were evenly distributed along the polypeptide chain they would be separated by about 130 amino acids. Provided that only thiol groups of the same polypeptide chain are interconnected, relatively frequent cross-bridge formation shows that the cysteine residues involved have either a much smaller intrachain distance or that thiol groups along the chain are brought close to each other by folding of the chain. In addition, however, interconnexions may be formed, not only between thiol groups of the same peptide chain, but also between neighbouring protein molecules (Simon & Konigsberg, 1966).

In spite of the occurrence of numerous cross-bridges, the change in the activity pattern of the catecholamine vesicles is not remarkably different from that produced by monofunctional blockage. If more than 30% of the thiol groups available are blocked by NEM or by BME, the catecholamine efflux increases considerably. This change is presumably caused by a loosening of the structure of the membranal protein, as is inferred from the observation that BME, NEM or the mercurial salyrgan dissolve the vesicles if the extent of blockage of the thiol groups becomes high. The

dissolution of the membranal protein is revealed by a large decrease of the turbidity of the vesicular suspension.

These results suggest that the interaction of thiol groups with other functional groups in the membranes is important for its stability. Disulphide bridges may also be involved, since 0.1 M-mercapto-ethanol produces the same clearing effect.

The structural change related to the increase of the catecholamine efflux seems not to be related to the inhibition of the ATP-dependent catecholamine influx produced under the same conditions by the thiol reagent. At lower extents of reaction of the thiol groups the catecholamine influx is much more affected than is the efflux. Whereas at the lowest extent of reaction (1.5 mol of thiol groups/ 10^6 g of protein) the influx is decreased by 60%, the efflux is not enhanced but significantly diminished. This decrease remains unexplained. It corresponds to the decrease in the efflux produced by low concentrations of reserpine (Taugner & Hasselbach, 1966). Further, although the influx can be protected by ATP to a high degree against the inhibiting effect of NEM and to a somewhat smaller extent against the inhibiting effect of BME, the efflux of the catecholamines is never significantly protected.

The vesicular adenosine triphosphatase that is protected against the inhibiting effect of BME, as has been found for NEM, comprises less than 20% of the total activity of the preparation. The maintenance of the correlation between the catecholamine influx and the protected adenosine triphosphatase activity in BME-treated preparations supplements the results obtained with NEM and supports the suggestion that the catecholamines are stored by an ATP-driven pump located in the vesicular membrane (Taugner & Hasselbach, 1968).

With regard to the specific involvement of thiol groups in catecholamine uptake it has been shown that there is no clear-cut relationship between the number of thiol groups that have reacted and the transport activity of the system. Thus the blockage of 1.5–2 mol of thiol groups/ 10^6 g of protein in the absence of ATP produces a severe inhibition, whereas after the blockage of 2–2.5 mol of thiol groups/ 10^6 g of protein in the presence of ATP the catecholamine influx remains fully active. If ATP prevents the blockage of essential thiol groups, as must be assumed, this effect is obscured by the continuing blockage of irrelevant groups.

As to the protection exerted by ATP two explanations seem plausible. First, ATP may compete directly with the thiol reagents. In this case it is attractive to assume that the competition may take place at the site where the thiol reagents are bound reversibly before they react irreversibly (see above). Secondly, ATP may protect by more indirect means, as observed for other membranes:

for instance, a structural transformation converting a relatively loose structure into a more rigid one (François, 1968). In the latter state thiol groups that may be directly or indirectly involved in the transport process can be attacked by none of the thiol reagents. Our finding that the formation of cross-bridges between neighbouring thiol groups is scarcely more effective than individual blockage shows that positional changes of these groups are not relevant for the catecholamine transport and the adenosine triphosphatase activity. Yet, if conformational changes do occur during catecholamine transport the size of the unit of the membrane protein involved can be estimated. It is assumed that each unit is connected with its neighbours by two BME-bridges. Each unit must therefore contribute 2 thiol groups to cross-bridge formation. Since 2mol of thiol groups/10⁶g of the total protein (which corresponds to approx. 10mol of thiol groups/10⁶g of membrane protein) can be blocked in the presence of ATP without affecting the activity (cf. Table 1 and Fig. 1), the size of the membrane protein unit possessing two thiol groups is 200000 daltons. Conformational changes are therefore likely to take place inside these units during catecholamine transport.

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