Organization of Thiol Groups of Electric-Eel Electric-Organ Sodium-plus-Potassium Ion-Stimulated Adenosine Triphosphatase Studied with Bifunctional Reagents

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The reactions of three bifunctional thiol-blocking reagents of differing cross-linking spans and two monofunctional thiol-blocking reagents with the $\text{Na}^+ + \text{K}^+$ -stimulated ATPase of the electric-eel electric organ were examined. 1,5-Difluoro-2,4-dinitrobenzene with a cross-linking span of 0.3–0.5 nm (3–5 Å) and high solubility in non-polar solvent was the most efficient inhibitor of enzyme activity; thus essential thiol groups exist in a non-polar environment and are approx. 0.3–0.5 nm (3–5 Å) from their nearest thiol-group neighbours. Ligands promoting phosphorylation of the $\text{Na}^+ + \text{K}^+$ -stimulated ATPase decreased the number of thiol groups bridged by 1,5-difluoro-2,4-dinitrobenzene and by 4,4'-difluoro-3,3'-dinitrodiphenyl sulphone [0.7–1.0 nm (7–10 Å) span]. Phosphorylation is associated with a conformational change in the enzyme.

Binding of the ligands ATP, Mg^{2+} , Na^+ and K^+ causes conformational modifications in $\text{Na}^+ + \text{K}^+$ -ATPase, and these have been detected in part by fluorescence (Karlsh *et al.*, 1977; Karlsh & Yates, 1978) and calorimetric (Kuricki *et al.*, 1976) techniques. The existence of these conformational changes is supported by studies with monofunctional reagents such as *N*-ethylmaleimide and 5,5'-dithiobis-(2-nitrobenzoic acid). They have shown that functionally different thiol groups are available for reaction with reagent in the presence of different ligands (Hart & Titus, 1973; Schoot *et al.*, 1978; Banerjee *et al.*, 1972; Harris & Stahl, 1976, 1977).

The enzyme contains approx. 35–40 mol of SH/mol of enzyme (M_r 250 000) measured in the presence of sodium dodecyl sulphate, and these can be divided into at least three populations on the basis of their reactivity towards thiol-blocking reagents (Schoot *et al.*, 1978). Approx. 18–22 mol of SH/mol of purified electric-eel $\text{Na}^+ + \text{K}^+$ -ATPase can be detected with 5,5'-dithiobis-(2-nitrobenzoic acid) in the absence of sodium dodecyl sulphate, and these are called 'readily available thiol groups' in the present paper. Within this population, about two to six thiol groups/molecule are essential for $\text{Na}^+ + \text{K}^+$ -ATPase activity (Hart & Titus, 1973). The use of bifunctional thiol-blocking reagents of differing cross-linking spans provides an approach for estimating

the distance between thiol groups and for assessing the effects of ligands such as ATP on these distances (Burke & Reisler, 1977). In the studies reported below we used changes in enzyme activity and thiol content to monitor bridging of thiol groups within the active centre of the $\text{Na}^+ + \text{K}^+$ -ATPase.

Materials and Methods

$\text{Na}^+ + \text{K}^+$ -ATPase was purified from the electric organ of the electric eel (*Electrophorus electricus*) by the procedure of Cantley *et al.* (1978). We excluded dithiothreitol from the second sucrose gradient and from subsequent steps of the procedure to eliminate exogenous thiols from the preparation. The isolated preparations had specific activities of 900–1200 μmol of P_i formed/h per mg of protein and were estimated to be greater than 90% pure on the basis of analysis after polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Harris & Stahl, 1977). $\text{Na}^+ + \text{K}^+$ -ATPase activity was assayed as previously described (Harris & Stahl, 1977), and protein was measured by the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard.

We determined thiol groups by the procedure of Boyne & Ellman (1972), utilizing 5,5'-dithiobis-(2-nitrobenzoic acid) with decolorization of reaction product to correct for light-scattering. Bifunctional reagents in acetone were added to enzyme suspensions so that the final acetone concentration did

Abbreviation used: $\text{Na}^+ + \text{K}^+$ -ATPase, $\text{Na}^+ + \text{K}^+$ -stimulated ATPase.

not exceed 5% (v/v). For determination of bridging of thiol groups, enzyme content of thiol groups was determined before and after incubation in the presence of the bifunctional reagents. For example, in Table 2, column (A), 20.2 mol of SH/mol of enzyme (M_r 250 000) was initially present, and after reaction with 1,5-difluoro-2,4-dinitrobenzene only 9.53 mol of SH/mol remained, so that 10.67 mol of thiol groups/mol were bridged. No free bifunctional reagent remained after the incubation with the $\text{Na}^+ + \text{K}^+$ -ATPase, i.e. each end had reacted with a thiol group. This reaction was established by the addition of known amounts of 2-mercaptoethanol to the enzyme after treatment with bifunctional reagent followed by assay for thiol content.

We determined partition coefficients of thiol-blocking reagents after shaking equal volumes of reagent in 150 mM-Tris/HCl buffer, pH 7.4, with iso-octanol for 20 min. The concentration of reagent in the aqueous phase was calculated from absorption values obtained at the following wavelengths: *p*-*NN'*-phenylenedimaleimide, 266 nm; 1,5-difluoro-2,4-dinitrobenzene, 260 nm; 4,4'-difluoro-3,3'-dinitrodiphenyl sulphone, 255 nm; 1-fluoro-2,4-dinitrobenzene, 260 nm; *N*-ethylmaleimide was measured by the method of Schoot *et al.* (1978).

Results and Discussion

Inhibition of $\text{Na}^+ + \text{K}^+$ -ATPase in the absence of ligands

Before using bifunctional reagents for examining spatial separation of thiol groups, we studied their

specificity for inhibiting enzyme activity. As shown in Fig. 1(b), the patterns of inhibition by the three bifunctional thiol-blocking reagents towards the $\text{Na}^+ + \text{K}^+$ -ATPase were similar to those of the monofunctional reagents (Fig. 1a). In relative effectiveness as inhibitors the reagents were in the order 1,5-difluoro-2,4-dinitrobenzene > 1-fluoro-2,4-dinitrobenzene > 4,4'-difluoro-3,3'-dinitrodiphenyl sulphone > *N*-ethylmaleimide > *p*-*NN'*-phenylenedimaleimide, estimated from the molar ratio of reagent to thiol groups sufficient to inhibit enzyme activity by 50%. 1,5-Difluoro-2,4-dinitrobenzene had the shortest span [0.3–0.5 nm (3–5 Å)] between reactive groups and was the most effective inhibitor; whereas *p*-*NN'*-phenylenedimaleimide had the longest span [1.2–1.4 nm (12–14 Å)] and was the least effective. The effectiveness of these reagents as inhibitors may depend on relative chemical reactivity, partition of reagent between non-polar and polar phases, and, with the bifunctional reagents, to the separation of reactive groups on the reagent molecule.

We examined the relative chemical reactivity of the reagents towards the thiol group in 2-mercaptoethanol (Table 1). From these data one would predict that *N*-ethylmaleimide and *p*-*NN'*-phenylenedimaleimide would be the best inhibitors of the $\text{Na}^+ + \text{K}^+$ -ATPase, whereas they were actually the least effective. Likewise, *p*-*NN'*-phenylenedimaleimide, with the longest spatial separation of reactive groups, might have the greatest probability of reacting with an essential thiol group within the active centre of the enzyme. This reasoning follows,

Fig. 1. Inactivation of $\text{Na}^+ + \text{K}^+$ -ATPase by thiol-blocking reagents

Electric-eel $\text{Na}^+ + \text{K}^+$ -ATPase (200 μg) in 150 mM-Tris/HCl buffer, pH 7.4, was incubated for 90 min at 25°C with (a) monofunctional or (b) bifunctional reagents at the indicated molar ratios. Reaction was terminated by 100-fold dilution with Tris/HCl buffer at 0°C and the enzyme was immediately assayed. The results of a single representative experiment are shown. (a) $\circ$, 1-Fluoro-2,4-dinitrobenzene (I); $\bullet$, *N*-ethylmaleimide (II). (b) $\square$, 1,5-difluoro-2,4-dinitrobenzene (III); $\blacksquare$, 4,4'-difluoro-3,3'-dinitrodiphenyl sulphone (IV); $\blacktriangle$, *p*-*NN'*-phenylenedimaleimide.

Table 1. *Properties of thiol-blocking reagents*

Relative reactivity was determined after mixing each reagent with 250 μ M-2-mercaptoethanol at pH 7.4 at 25°C in either a 1:1 molar ratio of reagent to thiol groups (monofunctional reagents) or a 0.5:1 molar ratio (bifunctional reagents). The rate of reaction was determined by withdrawal of samples at intervals and analysis for thiol content by the Boyne & Ellman (1972) procedure. The half-time for disappearance of thiol was determined in three separate experiments, and values relative to *N*-ethylmaleimide are reported. The half-time for disappearance of *p*-*NN'*-phenylenedimaleimide was less than 4 s and only an approximate relative activity was obtained.

Reagent	Cross-linking span [nm (Å)]	Partition coefficient (iso-octanol/buffer)	Relative reactivity
<i>N</i> -Ethylmaleimide	—	0.07	1
1-Fluoro-2,4-dinitrobenzene	—	1.09	0.0013
1,5-Difluoro-2,4-dinitrobenzene	0.3–0.5 (3–5)	0.41	0.0051
4,4'-Difluoro-3,3'-dinitrodiphenyl sulphone	0.7–1.0 (7–10)	0.38	0.0003
<i>p</i> - <i>NN'</i> -Phenylenedimaleimide	1.2–1.4 (12–14)	0.13	2–3 (approx.)

since, after reaction of the first end of a bifunctional reagent with a thiol group, the second reactive end is limited to interacting with those thiol groups within a volume element determined by the span between the reactive groups. Again, this did not appear to be the determining factor, since 1,5-difluoro-2,4-dinitrobenzene, with the shortest span, was the most effective inhibitor. We conclude that relative chemical reactivity and the spatial separation of groups within the reagents are less important than the third factor, partition coefficient. We found that the reagents 1,5-difluoro-2,4-dinitrobenzene and 1-fluoro-2,4-dinitrobenzene, with high partition coefficients, inhibited best, so that the thiol groups essential for enzyme activity exist in a non-polar environment, as had been found in previous studies on the $\text{Na}^+ + \text{K}^+$ -ATPase with a fluorescent probe for thiol groups (Harris & Stahl, 1977).

Organization of thiol groups and the effects of ligands

The enzymic mechanisms of the $\text{Na}^+ + \text{K}^+$ -ATPase involves several partial reactions and at least two phosphorylated intermediates:

and conversion of $\text{E}_1\text{-P}$ into $\text{E}_2\text{-P}$ involves thiol groups (Fahn *et al.*, 1966). We have studied the distribution of thiol groups in the $\text{Na}^+ + \text{K}^+$ -ATPase in the presence of ligands promoting formation of $\text{E}_1\text{-P}$ (ATP, Mg^{2+} , Na^+) and $\text{E}_2\text{-P}$ (Mg^{2+} , P_i), since these two phosphorylated forms may represent different conformational states of the enzyme (Banerjee *et al.*, 1972). The number of thiol groups/molecule readily available to 5,5'-dithiobis-(2-nitrobenzoic acid) did not significantly differ initially between the condition in which buffer alone was present or that in which ligands promoting

phosphorylation were present, as shown in Table 2. We added bifunctional reagents to enzyme in the ratio 0.5 mol/mol of SH, i.e. one functional group of reagent per thiol group, to minimize reaction without bridging of enzyme thiol groups. In each of these situations, *p*-*NN'*-phenylenedimaleimide bridged the largest number of thiol groups (66%) and inhibited about 80% of the enzyme activity. 1,5-Difluoro-2,4-dinitrobenzene, with the shortest span, bridged 43–52% of the total available thiol groups and inhibited enzyme activity by 98%. Bridging by 4,4'-difluoro-3,3'-dinitrodiphenyl sulphone was not significantly different from that by 1,5-difluoro-2,4-dinitrobenzene but was much less effective in inhibiting enzyme activity. These cross-linking experiments show that 1,5-difluoro-2,4-dinitrobenzene is most effective in bridging the essential thiol groups and in inhibiting $\text{Na}^+ + \text{K}^+$ -ATPase activity; it appears that the essential thiol groups exist in a non-polar region and are approx. 0.3–0.5 nm (3–5 Å) from their nearest thiol-group neighbours.

The number of thiol groups/molecule bridged by 1,5-difluoro-2,4-dinitrobenzene in the presence of $\text{ATP}/\text{Mg}^{2+}/\text{Na}^+$ apparently differed from that in

$\text{Mg}^{2+}/\text{P}_i$ or in buffer alone. It was estimated that one to two fewer thiol groups/molecule were bridged by this reagent when $\text{ATP}/\text{Mg}^{2+}/\text{Na}^+$ were present ($P < 0.006$). Likewise, one to three fewer thiol groups/molecule ($P < 0.02$) were spanned by 4,4'-difluoro-3,3'-dinitrodiphenyl sulphone in the presence of these same ligands. It would appear that fewer thiol groups/molecule are available for cross-linking by 1,5-difluoro-2,4-dinitrobenzene and 4,4'-difluoro-3,3'-dinitrophenyl sulphone in the $\text{E}_1\text{-P}$ state than in the E_1 state. No clear difference in the number of thiol groups/molecule bridged between

Table 2. *Effects of ligands on thiol groups bridged by bifunctional reagents*

Electric-eel $\text{Na}^+ + \text{K}^+$ -ATPase was suspended in (A) 150 mM-Tris/HCl buffer (pH 7.4) or (B) 40 mM-Tris/HCl buffer (pH 7.4)/100 mM-NaCl/5 mM- MgCl_2 /2 mM-ATP or (C) 140 mM-Tris/HCl buffer (pH 7.4)/5 mM- MgCl_2 /2 mM- P_i to a final concentration of 0.7 mg of protein/mg. Samples were withdrawn for determination of total thiol by the Boyne & Ellman (1972) procedure. Stoichiometric amounts of reagent (0.5 mol of reagent/mol of enzyme SH) were added, the mixture was kept at 25°C for 90 min, and $\text{Na}^+ + \text{K}^+$ -ATPase activity and thiol content were determined. Thiol groups bridged were calculated as described in the Materials and Methods section. The values reported $\pm$ s.d. were obtained from three enzyme preparations and each value represents at least four individual measurements.

Reagent	(A) Buffer		(B) Buffer/ $\text{Na}^+/\text{Mg}^{2+}/\text{ATP}$		(C) Buffer/ $\text{Mg}^{2+}/\text{P}_i$	
	No. of SH groups/molecule bridged	$\text{Na}^+ + \text{K}^+$ -ATPase activity (%)	No. of SH groups/molecule bridged	$\text{Na}^+ + \text{K}^+$ -ATPase activity (%)	No. of SH groups/molecule bridged	$\text{Na}^+ + \text{K}^+$ -ATPase activity (%)
None	0	100	0	100	0	100
1,5-Difluoro-2,4-dinitrobenzene	10.67 ± 1.01	2.5 ± 4.0	8.37 ± 0.67	2.4 ± 0.9	9.34 ± 1.46	2.3 ± 2.3
4,4'-Difluoro-3,3'-dinitrodiphenyl sulphone	9.62 ± 1.46	28.1 ± 1.8	7.06 ± 1.12	41.0 ± 1.7	7.13 ± 2.13	31.0 ± 1.7
<i>p</i> -NN'-Phenylenedimaleimide	13.31 ± 1.57	19.1 ± 2.8	12.42 ± 0.45	21.0 ± 8.9	12.62 ± 3.36	16.4 ± 2.5
Initial readily available SH (mol/mol of enzyme)	20.20 ± 0.70		18.45 ± 1.34		21.60 ± 4.14	

$\text{E}_1\text{-P}$ and $\text{E}_2\text{-P}$ and the non-phosphorylated form was established from the present data.

These studies strongly suggest that phosphorylation of the enzyme causes conformational modification involving the enzyme's thiol groups. However, the distance between essential thiol groups and adjacent thiol groups appears to remain about 0.3–0.5 nm (3–5 Å) even after phosphorylation of the enzyme to the $\text{E}_1\text{-P}$ state. 1,5-Difluoro-2,4-dinitrobenzene and 4,4'-difluoro-3,3'-dinitrophenyl sulphone, but not *p*-NN'-phenylenedimaleimide, should be useful in further exploration of spatial relationships between thiol groups in different conformational states of the enzyme.

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References

- Banerjee, S. P., Wong, S. M. E. & Sen, A. K. (1972) *Mol. Pharmacol.* **8**, 18–29
- Boyne, A. F. & Ellman, G. L. (1972) *Anal. Biochem.* **46**, 639–653
- Burke, M. & Reisler, E. (1977) *Biochemistry* **16**, 5559–5563
- Cantley, L. C., Gelles, J. & Josephson, L. (1978) *Biochemistry* **17**, 418–425
- Fahn, S., Hurley, M. R., Koval, G. J. & Albers, R. W. (1966) *J. Biol. Chem.* **241**, 1890–1895
- Harris, W. E. & Stahl, W. L. (1976) *Biochim. Biophys. Acta* **426**, 325–334
- Harris, W. E. & Stahl, W. L. (1977) *Biochim. Biophys. Acta* **485**, 203–214
- Hart, W. H. & Titus, E. O. (1973) *J. Biol. Chem.* **248**, 4674–4681
- Karlish, S. J. D. & Yates, D. W. (1978) *Biochim. Biophys. Acta* **527**, 115–130
- Karlish, S. J. D., Yates, D. W. & Glynn, I. M. (1977) *Nature (London)* **263**, 251–253
- Kuricki, Y., Halsey, J., Biltonen, R. & Racker, E. (1976) *Biochemistry* **15**, 4956–4961
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Schoot, B. M., DePont, J. J. H. M. & Bonting, S. L. (1978) *Biochim. Biophys. Acta* **522**, 602–613