Conformational states of the (Na⁺ + K⁺)-transporting ATPase

Formation of 240000-*M_r* and 116000-*M_r* polypeptides in the presence of a bifunctional thiol probe

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1. Interpeptide cross-linking of α -subunits with concomitant loss of Na⁺ + K⁺-transporting ATPase (Na⁺,K⁺-ATPase) activity was found when the purified lamb kidney enzyme was treated with the bifunctional thiol reagent 4,4'-difluoro-3,3'-dinitrodiphenyl sulphone (F₂DNS). Several forms of the enzyme could be clearly distinguished: one binding ATP (non-phosphorylated enzyme, E₁·ATP), a phosphorylated form (E₂-P) and a phosphoenzyme-ouabain complex (E₂P·ouabain). 2. A polypeptide of approx. *M_r* 240000 and probable α_2 composition comprised up to 5–20% of the total polypeptides after reaction of the lamb kidney Na⁺,K⁺-ATPase with F₂DNS. The amount of this polypeptide formed was related to the conformational state of the enzyme. The presence of adenine nucleotide greatly diminished the amount of 240000-*M_r* polypeptide formed and provides evidence for an enzyme-adenine-nucleotide complex under conditions where the enzyme is not phosphorylated. 3. F₂DNS reacted with the enzyme in the presence of Mg²⁺, P_i and ouabain to form a new polypeptide with an approx. *M_r* of 116000, and comprised 23% of the total, whereas the 240000-*M_r* polypeptide comprised 9% of the total. This suggests that the 116000-*M_r* polypeptide is a characteristic marker of the E₂P·ouabain complex. By using specific antibodies it was established that both the 240000- and 116000-*M_r* polypeptides contained α -, but not β -, subunits of the Na⁺,K⁺-ATPase.

(Na⁺ + K⁺)-transporting-ATPase (Na⁺,K⁺-ATPase), the enzyme mediating active Na⁺ and K⁺ transport in cells, is composed of at least two non-covalently associated subunits. These are the α -subunit, having an *M_r* of approx. 95000–100000 and containing the catalytic site, and the β -subunit, a glycopeptide with an *M_r* of approx. 45000–50000. In some preparations a lower-*M_r* proteolipid, sometimes designated the 'γ-peptide', has also been observed (Forbush *et al.*, 1978; Rogers & Lazdunski, 1979*a,b*; Reeves *et al.*, 1980; Collins *et al.*, 1982). The presence of the latter peptide has not yet been documented in all purified Na⁺,K⁺-

ATPase preparations, and its present status remains tenuous.

The native enzyme, isolated from a number of sources, contains approx. 45–52 mol of thiol groups/mol of enzyme (320000 *M_r*) (Harris & Stahl, 1980; Schoot *et al.*, 1978), and, on the basis of experiments using thiol-group-blocking reagents, about two to six thiol groups/enzyme molecule are required for full enzymic activity (Hart & Titus, 1973). Thiol groups reside primarily on the α -subunit of the enzyme, and the availability of functionally different thiol groups to blocking reagents is modified by the conformational state of the enzyme (Harris & Stahl, 1980; Patzelt-Wenczler & Schoner, 1981). Several studies (Craig & Kyte, 1980; Askari *et al.*, 1980) have demonstrated that the α - and β -subunits may be linked covalently by bifunctional reagents under conditions favouring a specific conformation of the enzyme. For example, Huang & Askari (1981) used copper-phenanthroline to catalyse formation of disulphide

Abbreviations used: Na⁺,K⁺-ATPase, (Na⁺ + K⁺)-transporting ATPase; SDS, sodium dodecyl sulphate; F₂DNS, 4,4'-difluoro-3,3'-dinitrodiphenyl sulphone; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); K⁺-pNPPase, K⁺-stimulated *p*-nitrophenyl phosphatase; IgG, immunoglobulin G.

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bonds in the Na^+, K^+ -ATPase and found cross-linking (α_2) subunits only when the enzyme was in a conformation that permitted phosphorylation. In contrast, Craig & Kyte (1980) reported that the higher- M_r species found after copper-phenanthroline treatment was a heterodimer of $\alpha\beta$ composition.

The present study was undertaken to examine further the organization of the subunits in different conformational states by cross-linking available thiol groups. The enzyme was allowed to react in the presence of ligands that promote specific conformational states of the enzyme (Harris & Stahl, 1980) with F_2DNS , a bifunctional thiol reagent (Burke & Reisler, 1977) having a span of 0.7–0.9 nm between reactive ends.

Materials and methods

The Na^+, K^+ -ATPase was purified from lamb kidney outer medulla by the procedure of Lane *et al.* (1979). It had an initial specific activity of between 1400 and 1700 $\mu\text{mol/h}$ per mg of protein, which slowly declined with storage at 2–4°C over a period of several months. This preparation bound 1700–2100 pmol of [^3H]ouabain/mg of protein and formed 1800–2000 pmol of [γ - ^{32}P]phosphoenzyme intermediate/mg of protein (see the following paper, Harris & Stahl, 1984).

Several ligand conditions were used in different parts of the present study to modify the enzyme conformation. Ligands were used in combinations to be approximately equivalent in ionic strength to 200 mM-HCl, pH 7.4. The concentration of Tris/HCl was adjusted accordingly in each case. The concentrations of other ligands were as follows: 100 mM-NaCl; 25 mM-KCl; 5 mM- MgCl_2 ; 3 mM-ATP (unless stated otherwise); 3 mM- P_i ; 0.10 mM-ouabain; oligomycin, approx. 10 μM . Thus the $\text{MgCl}_2/\text{Tris}$ solution was 5 mM- MgCl_2 , 190 mM-Tris/HCl; $\text{MgCl}_2/\text{KCl}/\text{P}_i/\text{Tris}$ was 5 mM- MgCl_2 , 25 mM-KCl, 3 mM- P_i and 162 mM-Tris/HCl. In some cases [γ - ^{32}P]ATP (usually 6×10^5 c.p.m./ml) was added to the reaction mixtures in order to monitor hydrolysis of ATP. For uniformity, F_2DNS was dissolved in dimethyl sulphoxide and a small portion (less than 5% total volume) was added to the medium containing the 0.5 mg of enzyme/ml of suspension. Dimethyl sulphoxide was added to a control preparation run in parallel with those samples treated with bifunctional reagents. A molar ratio of 0.6 mol of bifunctional reagent (1.2 mol of thiol-reactive species) to one enzyme thiol group was used to correct for the possible solvolysis of the reagent, and the reaction was carried out for up to 240 min at 37°C. This was terminated by the addition of a twofold molar

excess of β -mercaptoethanol, followed by centrifugation. Thiol was removed by washing the enzyme preparation three times (30 000g for 20 min each time) in ice-cold 200 mM-Tris/HCl, pH 7.4. Thiol content was determined by the procedure of Boyne & Ellman (1972), as previously described (Harris & Stahl, 1980), by using Nbs_2 . [^{32}P]ATP hydrolysis was monitored by using standard techniques [see the following paper (Harris & Stahl, 1984) and Stahl (1973)].

SDS/polyacrylamide-slab-gel electrophoresis was performed as described by Laemmli (1970) on gradient gels. A gradient resolving gel (6–8% acrylamide, pH 8.5) was used with a 5% stacking gel (pH 6.5). Approx. 10 μg of each sample in a reducing buffer (Laemmli, 1970) was applied per lane, and the electrophoresis was run at 4°C at 20 mA/gel until the Bromophenol Blue tracking dye was near the bottom of the resolving gel. The gels were stained with Coomassie Blue dye in acetic acid/propan-2-ol/water (10:7:83, by vol.) and destained in 10% (v/v) acetic acid. For apparent- M_r estimation a plot of the logarithm of the migration distance of unknown samples was compared with that of M_r standards. Sample lanes were later cut out of the slab and scanned at 550 nm in a linear-transport attachment to a Gilford spectrophotometer and recorded. The areas under the peaks were integrated with a Graphicon instrument attached to a PDP-11 computer. Linearity between total area of Coomassie Blue-staining material was found in this gel system when 2–11 μg of protein was applied. Greater quantities of protein resulted in proportionally less staining and non-linearity. Cross-linking did not significantly alter the amount of sample that entered the gel.

The Na^+, K^+ -ATPase activity was assayed as previously described (Harris & Stahl, 1977) using a colorimetric method for phosphate determination (Lanzetta *et al.*, 1979). pNPPase activity with and without K^+ was assayed by a standard procedure (Harris & Stahl, 1977). Protein was analysed by the procedure of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Antisera

Antisera to the 95 000- M_r (α) and 50 000- M_r (β) polypeptides of the Na^+, K^+ -ATPase were obtained as follows. Lamb kidney Na^+, K^+ -ATPase (700 $\mu\text{g/gel}$) was separated into 95 000- M_r and 50 000- M_r polypeptides by SDS/polyacrylamide-gel electrophoresis using 7% (w/v) analytical gels essentially as described above. After electrophoresis, bands were located by staining with Coomassie Blue, destained in 10% (w/v) acetic acid and cut out and washed for 1–2 days in distilled water at room temperature and were stored frozen at –20°C until needed. Rabbits were injected sub-

cutaneously with polyacrylamide gel containing purified α - or β -polypeptides. In each case gel containing approx. 500 μg of protein was ground in a glass homogenizer with 1–2 ml of 0.9% NaCl and an equal volume of Freund's complete adjuvant. The same procedure was followed 1 month later, but Freund's incomplete adjuvant was used. Animals were bled at weekly intervals thereafter and were assayed for antibodies by using an enzyme-linked immunosorbent assay. The specificity of the antisera was established by using the immunoblotting technique.

The immunoblotting technique has been previously described (Schellenberg *et al.*, 1981) and was modified as follows. The antigen-antibody complex bound to nitrocellulose was incubated with peroxidase-conjugated goat anti-rabbit IgG (Cappel, Cochranville, PA, U.S.A.) diluted 1:1000 in a solution of 5% (w/v) bovine serum albumin, 0.15 M NaCl and 10 mM-Tris/HCl, pH 7.4, for 1 h at room temperature. The nitrocellulose sheet was washed three times (10 min each) in 0.15 M NaCl/10 mM-Tris/HCl, pH 7.4, and was placed in 50 ml of fresh buffer. To this was added 10 ml of 4-chloro-1-naphthol (3 mg/ml) in methanol and 50 μl of 30% H_2O_2 . After 5–10 min reaction, product could be clearly seen and the sheets were washed in fresh buffer and stored on absorbent paper sheets overnight for drying.

Results

Different ligands may modify the relative position of thiol groups within the structure of the Na^+, K^+ -ATPase and may change the accessibility of the bifunctional reagent to the reactive groups. The Na^+, K^+ -ATPase was allowed to react with the bifunctional thiol reagent F_2DNS in the presence of combinations of ligands known to modify the conformation of the enzyme. Untreated lamb kidney Na^+, K^+ -ATPase was electrophoresed in the presence of SDS to yield two major bands of M_r about 95 000 and 50 000 and minor bands (Fig. 1, lane 1), representing respectively, 66.8, 31.4 and 2.7% of the total Coomassie Blue-stained area occupied by polypeptides entering polyacrylamide gels (Table 1). In different experiments and preparations used in the present study the apparent M_r of the two major polypeptides have been between 92 000–96 000 and 47 000–50 000. In the present paper we have adopted values of 95 000 and 50 000 for the two major components. When the lamb kidney Na^+, K^+ -ATPase was treated with F_2DNS , new polypeptide bands were found with apparent M_r values of 240 000 and 116 000 (Fig. 1), comprising up to 21 and 25% respectively of the total polypeptide area, depending upon ligand conditions used (Table 1, columns A

Fig. 1. SDS/polyacrylamide-gel electrophoresis of Na^+, K^+ -ATPase treated with the bifunctional reagent F_2DNS

The enzyme was treated with bifunctional reagent as described in the Materials and methods section and between 10 and 12 μg of protein was applied to each well (6–8% gradient gel) and electrophoresed at a current of 10 mA/gel. The gel was stained with Coomassie Blue dye. In lanes 2–8 the enzyme was allowed to react with bifunctional reagent in Tris/HCl buffer in the following solutions (see the Materials and methods section for details): lane 2, no additions; lane 3, 100 mM- Na^+ ; lane 4, 5 mM- Mg^{2+} ; lane 5, 3 mM-ATP; lane 6, 100 mM- Na^+ , 5 mM- Mg^{2+} , 3 mM-ATP; lane 7, 100 mM- Na^+ , 5 mM- Mg^{2+} , 3 mM-ATP, 10 μM -oligomycin; lane 8, 5 mM- Mg^{2+} , 3 mM- P_i , 0.1 mM-ouabain. The lamb kidney enzyme was included as a control in lane 1 in the absence of bifunctional reagent. Bio-Rad high- and low- M_r standards were run in the outer lanes (not shown) and the arrows in the left margin indicate the positions of these standard proteins. The asterisk (*) to the right identifies the 116 000- M_r polypeptide. Also, on the right margin, the α - and β -subunits are indicated, whereas the upper and lower arrows identify the 240 000- and 12 000- M_r polypeptides respectively. The latter polypeptide generally comigrates with the tracking dye.

and B). To determine the composition of the multimers formed from Na^+, K^+ -ATPase subunits in the presence of the cross-linking agent copper-phenanthroline, Craig & Kyte (1980) measured the proportional decrease in area of the α - and β -polypeptides on polyacrylamide gels. We used the same technique and found that the sum (column D, Table 1) of the areas for the cross-linked polypeptide (columns A and B, Table 1) and 95 000- M_r polypeptide (α -subunit, column C) was approxi-

Table 1. Polypeptide composition of lamb kidney $\text{Na}^+, \text{K}^+ \text{-ATPase}$ after cross-linking with 4,4'-difluoro-3,3'-dinitrodiphenyl sulphone

Cross-linking in the presence of F_2DNS was carried out for 4 h as described in the Materials and methods section. Ligand concentrations were as follows: 100 mM- NaCl ; 25 mM- KCl ; 5 mM- MgCl_2 ; 3 mM-ATP (unless indicated otherwise); 3 mM- P_i ; 0.1 mM-ouabain; approx. $10 \mu\text{M}$ -oligomycin. Polypeptides were then separated by SDS/polyacrylamide-gel electrophoresis on gradient (6–8%, w/v) polyacrylamide gels (approx. $10 \mu\text{g}$ of protein/ lane), stained with Coomassie Blue, destained, and the gels were scanned at 550 nm. The total area under all peaks was computed, excluding material that did not enter the stacking gel, and the contribution of each peak area to the total stained area was calculated. The reproducibility of the procedure for three to four scans and integrations of a single sample lane was $\pm 5\%$. In a given experiment approximately the same amount of polypeptide entered each lane, on the basis of the total integrated areas. The other samples were run at least in duplicate, and mean values ($\pm \text{S.D.}$) are reported.

Ligands	n	Polypeptide ...	Percentage of total area for each polypeptide					
			A	B	C	D	E	F
			240 000- M_r	116 000- M_r	95 000- M_r	(A + B + C)	50 000- M_r	Minor bands
Control enzyme (no ligands, no F_2DNS)	14		0	0	66.8 ± 3.1	66.8	31.4 ± 3.8	2.7 ± 1.5
None	8		20.5 ± 2.3	0	34.3 ± 1.8	59.5	36.5 ± 5.6	6.2 ± 3.0
Na^+ or K^+ or Mg^{2+} or P_i	3		19.4 ± 2.9	0	38.7 ± 4.6	65.2	32.8 ± 5.2	9.2 ± 4.0
ATP (3 mM)	3		10.4 ± 6.6	0	47.7 ± 8.1	62.8	35.1 ± 10.6	8.0 ± 3.4
ATP (100 μM)	2		0	0	59.9	59.9	40.3	0
ATP, Na^+	3		10.1 ± 1.4	0	55.9 ± 3.3	66.0	28.9 ± 5.3	4.4 ± 0.4
ATP, K^+	3		4.6 ± 1.2	0	59.7 ± 1.6	65.5	29.7 ± 6.2	4.1 ± 2.4
ATP, Mg^{2+} , Na^+	9		8.3 ± 4.4	0	53.4 ± 5.9	67.8	33.2 ± 2.2	4.2 ± 2.2
ATP, Mg^{2+} , Na^+ , oligomycin	5		8.5 ± 5.0	0	52.2 ± 7.9	60.7	31.9 ± 3.6	3.9 ± 1.4
Mg^{2+} , P_i	4		8.5 ± 3.0	0	45.3 ± 8.5	56.5	37.0 ± 3.1	4.4 ± 1.5
Mg^{2+} , P_i , ouabain $\pm \text{K}^+$	3		8.7 ± 1.6	23.1 ± 3.2	34.8 ± 3.3	66.6	28.7 ± 2.8	3.9 ± 1.5
Ouabain	1		14.1	0	38.2	52.3	42.6	5.1
Oligomycin	3		18.2 ± 2.5	0	40.4 ± 5.4	58.6	41.3 ± 4.6	0

Fig. 2. Immunoreactivity of $\text{Na}^+, \text{K}^+ \text{-ATPase}$ polypeptides

Representative immunoblots on nitrocellulose sheets are shown and the procedure is described in the Materials and methods section. The original 6–8% gradient gel was overloaded by applying between 30 and $38 \mu\text{g}$ of protein, and this was run essentially as shown in Fig. 1. These samples were then transferred to nitrocellulose sheets. The sheets were treated with antibodies specific to either the α - (right sheet, b) or β - (left sheet, a) subunits of the enzyme. The samples applied are described in Fig. 1. The positions of the 240 000- M_r polypeptide (upper right arrow), the 116 000- M_r polypeptide (asterisk) and the α - and β -subunits are indicated.

Fig. 3. (a) Formation of cross-linked polypeptides and (b) ATP hydrolysis

Enzyme was treated with F₂DNS as described in the Materials and methods section. When ATP was present, the reaction mixture also contained 6×10^5 c.p.m. of [γ -³²P]ATP/ml. At the indicated times samples were withdrawn and analysed, where appropriate, for [³²P]P_i and other samples were centrifuged to isolate the F₂DNS-treated enzyme. The polypeptide composition was determined as described in the Materials and methods section. Results are from a single representative experiment. 240000-*M_r* polypeptide: ○, 100 μM-ATP; ●, 3 mM-ATP; □, 3 mM-ATP, 100 mM-Na⁺, 5 mM-MgCl₂, 10 μM-oligomycin; ◇, 3 mM-ATP, 100 mM-Na⁺, 5 mM-Mg²⁺, 25 mM-K⁺; ■, no ligands (Tris); △, 5 mM-Mg²⁺, 3 mM-P_i, 0.1 mM-ouabain; 116000-*M_r* polypeptide: ▲, 5 mM-Mg²⁺, 3 mM-P_i, 0.1 mM-ouabain.

Table 2. Summary of characteristics of Na⁺,K⁺-ATPase after treatment with bifunctional probe in different conformational states

Enzyme activities were compared with that of control enzyme (which was not treated with F₂DNS) and were as follows: very low, <10% of control; intermediate, 30–40% of control; –, not determined.

Ligand condition during treatment with probes
(characteristic level observed)

Parameter	Tris (E ₁)	Tris, ATP (E ₁ ·ATP)*	Mg ²⁺ , P _i (E ₂ -P)	Mg ²⁺ , P _i , Ouabain ± K (E ₂ -P·Ouabain)
240000- <i>M_r</i> polypeptide	High	Intermediate	Intermediate	Intermediate
116000- <i>M_r</i> polypeptide	Absent	Absent	Absent	Present
Na ⁺ ,K ⁺ -ATPase activity	Very low	Intermediate	Very low	–
K ⁺ -pNPPase activity	Very low	Intermediate	Low/intermediate	–

* No hydrolysis of 0.1 or 3 mM-[γ -³²P]ATP (see Fig. 3b).

mately equal to the percentage of the 95000-*M_r* polypeptide in the Na⁺,K⁺-ATPase before cross-linking (Table 1, column C). This suggests that the 240000-*M_r* multimer has an α₂ composition.

With the immunoblotting technique the Na⁺,K⁺-ATPase treated with bifunctional reagent was transferred to nitrocellulose sheets, incu-

bated with antisera specific to either the 95000-*M_r* or 50000-*M_r* polypeptides, and the antigen-antibody complexes were identified with peroxidase-conjugated goat anti-rabbit IgG (blue reaction product is formed when 4-chloro-1-naphthol is the chromogenic donor). As shown in Fig. 2, the antisera used were specific to either the 95000-*M_r* (Fig.

2b) or 50000- M_r (Fig. 2a) polypeptides of the untreated Na^+, K^+ -ATPase. The 240000- M_r polypeptide (Fig. 1), appearing after treatment with bi-functional reagent (Table 1), reacted only with the antiserum to the 95000- M_r polypeptide, so that the 240000- M_r polypeptide does not contain β -peptide.

A 116000- M_r band produced by reaction with F_2DNS only under certain ligand conditions has too small a M_r to be an $\alpha\beta$ -cross-linked product. The presence of the α -polypeptide and absence of β -polypeptide in this cross-linked species was confirmed by reaction with specific antisera (Fig. 2). But the 116000- M_r polypeptide could be a product of cross-linking the α -subunit and a lower- M_r species, perhaps the 12000- M_r polypeptide (Forbush *et al.*, 1978; Rogers & Lazdunski, 1979a,b; Collins *et al.*, 1982). This latter species runs very close to the dye front in most polyacrylamide-gel systems (Fig. 1, lower right arrow). It has been found in several preparations of the Na^+, K^+ -ATPase, although its role in the enzyme complex remains to be determined.

Cross-linking of polypeptide in different conformational states

We allowed the Na^+, K^+ -ATPase to react with F_2DNS under many different ligand conditions (Table 1) and found that the 240000- M_r polypeptide was almost always present, but in various proportions relative to the total polypeptide population. The time course for formation of the 240000- M_r polypeptide with several sets of ligands is shown in Fig. 3(a). A 4h incubation period was chosen for the present study, but may not be optimum for examining several of the states of the enzyme. After 4h incubation the proportion (approx. 20%) of the 240000- M_r polypeptide found with F_2DNS in the presence of 100mM- Na^+ or 25mM- K^+ or 5mM- Mg^{2+} or 3mM- P_i (Table 1) did not significantly differ from when enzyme was incubated with Tris buffer alone, and these data were combined in Table 1 for convenience. The presence of 3mM-ATP significantly lowered the 240000- M_r polypeptide content to 10.4% ($P < 0.003$, compared with the situation with Tris buffer alone). But in the presence of 100 μM -ATP, no cross-linked species could be detected. In both of these situations the enzyme was not phosphorylated, as indicated by lack of hydrolysis of [^{32}P]-ATP (Fig. 3b). Generally the 240000- M_r polypeptide content observed using all other combination of enzymes with 3mM-ATP and ligands shown in Table 1 was not significantly different from that obtained with 3mM-ATP alone. This lowering of the 240000- M_r polypeptide content (compared with that obtained with Tris buffer) was also achieved with 3mM-ADP or -AMP, but not by

3mM-ITP, -UTP or -GTP (results not shown). The combination of 3mM-ATP and 25mM- K^+ was even more effective than 3mM-ATP alone in modifying enzyme thiol groups, and lowered the 240000- M_r polypeptide content to 4.6%. This clearly differed from 3mM-ATP alone or 3mM-ATP plus 100mM- Na^+ ($P < 0.01$). These results suggest that binding of ATP or other adenine nucleotide, rather than phosphorylation of the Na^+, K^+ -ATPase, must elicit sufficient conformational change to account for the major observed differences in F_2DNS reaction and attendant changes in 240000- M_r polypeptide content. Experiments were also carried out with 3mM-ATP and 5mM- Mg^{2+} , but sufficient Na^+ and K^+ (measured by atomic-absorption spectrometry) were present so that ATP was hydrolysed within approx. 0.5h. This condition complicated interpretation of the effects of Mg^{2+} and ATP. Addition of 5–10mM-(1,2-cyclohexylenedinitrilo)tetra-acetate (CDTA) to complex endogenous Mg^{2+} in the presence of 3mM-ATP did not alter the polypeptide composition (results not shown), suggesting that little endogenous Mg^{2+} is present in the preparation.

We also carried out experiments with conditions favouring phosphorylated states (Table 1). As shown in Fig. 3(b) in experiments using ATP under phosphorylating conditions, even with oligomycin present to trap $\text{E}_1\text{-P}$ (Inturrisi & Titus, 1968; Stahl, 1968), up to 60% of the ATP had disappeared within the first hour. These conditions were probably insufficient to stabilize $\text{E}_1\text{-P}$ for long enough to allow optimum reaction of F_2DNS with the Na^+, K^+ -ATPase.

The $\text{E}_2\text{-P}$ intermediate is generally favoured in the presence of Mg^{2+} and P_i (Siegel *et al.*, 1969). By using these ligands, a relatively high level of phosphorylated enzyme could be formed [cf. following paper (Harris & Stahl, 1984), Table 1], but the 240000- M_r polypeptide composition (8.5%) was similar to that found in ATP alone (Table 1). However, ouabain appears to stabilize the phosphorylated state of the enzyme, possibly as E_2 -ouabain or more likely as $\text{E}_2\text{-P}$ -ouabain (Siegel *et al.*, 1969), and in lamb kidney the steady-state level of phosphorylated intermediate formed from [^{32}P] P_i and Mg^{2+} was increased by 30% in 0.1mM-ouabain [cf. the following paper (Harris & Stahl, 1984)]. The formation of this complex did not appreciably change the 240000- M_r polypeptide composition, compared with that obtained with ATP, but now a new polypeptide of M_r -116000 was observed (Table 1 and Fig. 1, lane 8). This latter polypeptide was a major polypeptide and comprised about a quarter of the stained polypeptides after 4h incubation with F_2DNS (Fig. 3a). It would appear that this cross-linked polypeptide is a good marker for

this ouabain-stabilized conformation of the Na⁺,K⁺-ATPase. K⁺ with Mg²⁺, P_i and ouabain gave similar results, and, as shown in the following paper (Harris & Stahl, 1984), should lead to about the same steady-state level of phosphorylation.

Thiol content and enzymic activity

After the reaction of the bifunctional reagent with the Na⁺,K⁺-ATPase, the thiol content and enzymic activity of the modified enzyme were examined to assess whether thiol groups, which are vital to Na⁺,K⁺-ATPase and K⁺-pNPPase enzyme activities, had been cross-linked. The loss of the Na⁺,K⁺-ATPase thiol groups after reaction with F₂DNS was about 27 thiol groups/mg of protein in the presence of any ligand combination; without ligands, 48 thiol groups/mg of protein were lost. No significant difference between ligand conditions was found.

The two enzymic activities measured indicate that the presence of most of the ligands has a protective effect on the Na⁺,K⁺-ATPase and the K⁺-pNPPase activity (Table 2). The presence and presumed binding of ATP, alone or in combination with other ligands, protects the active site of the enzyme from reaction with F₂DNS. Similarly, the presence of ATP with K⁺ protects the K⁺-pNPPase activity. The K⁺-pNPPase activity was higher when the F₂DNS reaction was run in the presence of K⁺ than with other ions (results not shown).

We interpret the data to indicate that the reaction with F₂DNS causes the same number of thiol groups to be cross-linked in all of these experimental situations with a variety of ligands. However, different individual thiol groups are involved in each of these cases, since different levels of enzymic activity were retained.

Discussion

The main finding that emerges from the present study is that the reaction of a bifunctional SH probe with the Na⁺,K⁺-ATPase, in the presence of specific ligands, permits one to distinguish several different conformations of the enzyme.

F₂DNS, which has a distance of 0.7–0.9 nm between reactive moieties, may form either intra- or inter-peptide bridges with the enzyme. The reaction of F₂DNS with the thiol groups of the Na⁺,K⁺-ATPase under all ligand combinations examined (except 100 μM-ATP) led to the formation of polypeptides of *M_r* greater than 100 000 that were combinations of the α-polypeptide. This was associated with loss of Na⁺,K⁺-ATPase activity and, to a lesser degree, with loss of K⁺-pNPPase activity. The better protection against cross-linking with 100 μM-ATP versus 3 mM-ATP suggests that the former involves a high-affinity binding

ATP site, whereas the latter involves both high- and low-affinity sites. It is unclear why 3 mM-ATP does not protect as well as 100 μM-ATP, since the higher concentration of ATP should saturate the high-affinity site. Possibly several E₁-adenine nucleotide forms of the enzyme are involved.

In the presence of any ligand combination, approx. 27 thiol groups/mg of protein were lost, presumably due to reaction with F₂DNS. This could form a maximum of 14 cross-links/mg of protein. Without ligands present, almost twice that number, 48 nmol/mg of protein, were lost, which could result in a maximum of 23 cross-links/mg of protein within the enzyme. It is most likely that most of the cross-linking is intrapeptide in nature. Interpeptide cross-linking did occur, as evidenced by the presence of higher-*M_r* species under all the conditions examined. Assuming an *M_r* of 320 000 for the Na⁺,K⁺-ATPase, there would be a maximum of 4.5 bridges per molecule of enzyme in the presence of ligands and 8.7 maximum potential bridges without ligands present during the reaction with F₂DNS. The changes in distribution of higher-*M_r* species was dependent upon this cross-linking.

Cross-linked polypeptides

The multimer band found on SDS/polyacrylamide gels with an apparent *M_r* of 240 000, after reaction of the lamb kidney Na⁺,K⁺-ATPase with F₂DNS, appears to be composed of two α-peptides, in agreement with Askari (1982), who used *o*-phenanthroline as the cross-linking reagent. This conclusion is based on: (1) reactivity of the 240 000-*M_r* polypeptide with specific antibodies to the α-, but not to the β-, polypeptides (Fig. 2), and (2) the proportional decrease in area of the polypeptide band after Coomassie Blue staining while the area of the β-band remained constant (Table 1). The apparent *M_r* of this cross-linked species appears to be greater than the sum of two individual α-peptides. However, the *M_r* of the α-polypeptide determined by Craig & Kyte (1980) was 125 000, so an α₂ dimer would be in the range found in the present study. Peters *et al.* (1981) determined an *M_r* for the α-peptide of 131 000 and for the β-peptide an *M_r* of 61 800, but Hastings & Reynolds (1979) reported values of 106 400 and 36 600 for the α- and β-polypeptides for the enzyme isolated from shark rectal gland. Craig & Kyte (1980) have argued that the Na⁺,K⁺-ATPase α-peptide migrates faster (lower apparent *M_r*) on SDS/polyacrylamide gels compared with *M_r* standards, owing to a higher proportion of aliphatic side chains in the enzyme than in the proteins used as standards. Substitution of hydrophobic amino acids for hydrophilic ones in polypeptides has been shown to increase electrophoretic mobility in SDS/polyacrylamide-gel electrophoresis (de Jong *et al.*, 1978). This may be

due to altered binding of SDS by the polypeptide. Cross-linking of α -subunits may modify the availability of such hydrophobic (or other) residues to SDS, so that a lower mobility for a cross-linked species would be observed. Generally, the estimation of M_r by SDS/polyacrylamide-gel electrophoresis is based on the binding of SDS to unfolded polypeptide in a random-coil configuration. Cross-linking or the formation of intrapeptide thiol bridges probably limits unfolding of the polypeptide and this would tend to decrease electrophoretic mobility. These uncertainties make it difficult to eliminate the participation of other smaller polypeptides, e.g. the γ -polypeptide of 12000 M_r in the 240000- M_r complex. This may be ascertained when antibodies to the γ -polypeptide become available. No clear correlation between loss of minor polypeptides (Table 1, column F) and the appearance of the 240000- M_r polypeptide is evident.

Our results differ from several previous reports in that we did not find the $\alpha\beta$ -heterodimer reported by Craig & Kyte (1980), but primarily multimers of the α -subunit as reported by Huang & Askari (1981). By using the bifunctional probe F_2 DNS we were unable to demonstrate exclusive cross-linking associated with phosphorylation of the enzyme. Rather, we found maximum cross-linking to form the 240000- M_r polypeptide in the absence of phosphorylation (Table 1). However, different preparations and a different cross-linking reagent were used in those studies, and as discussed in the accompanying paper (Harris & Stahl, 1984), availability of specific sites on the enzyme may vary substantially between different organs and species.

An unexpected finding with the use of F_2 DNS was that in addition to the occurrence of the 240000- M_r polypeptide, another protein of apparent M_r 116000 was found. The 116000- M_r band could be one of two obvious combinations of the known peptides in the Na^+, K^+ -ATPase preparation. First, the apparent M_r is such that it could be at least a β_2 multimer. However, the immunoblot experiments eliminate this possibility. Another possibility would be that this species is composed of an $\alpha\gamma$ cross-linked peptide. The γ -polypeptide with an approx. M_r of 12000 is an acidic proteolipid of unknown function in the Na^+, K^+ -ATPase. Resolution and quantification of the γ -peptide, however, are poor and need additional study. Another explanation would be that the 116000- M_r polypeptide is the α -peptide locked into a different conformation by reaction with F_2 DNS. In this conformation SDS may have restricted ability to bind with amino acid side chains of the peptide, lowering its mobility on SDS/polyacrylamide gels. This was discussed above with regard to the 240000- M_r polypeptide.

The formation of the 116000- M_r polypeptide is of special interest in view of the findings of Rogers & Lazdunski (1979a,b) and Forbush *et al.* (1978). These workers found that a radioactive photo-affinity ouabain probe labelled both the 95000- M_r and 12000- M_r polypeptides under conditions expected to produce phosphorylated enzyme. The present experiments confirm the presence of the 95000- M_r component in the 116000- M_r multimer. If the 12000- M_r polypeptide is also a part of this multimer, at least one thiol group reactive with F_2 DNS must be present on this polypeptide as well as on the 95000- M_r polypeptide when the enzyme is in the E_2 -P ouabain state. The pair must be within 0.7–0.9 nm for the cross-linking to occur. The characteristics of this complex may better define how ouabain modifies the structure of the Na^+, K^+ -ATPase and deserves further study.

Conformations of the Na^+, K^+ -ATPase

The observed characteristics of selected states of the enzyme after F_2 DNS treatment are summarized in Table 2. E_1 and E_2 are the forms of the enzyme (Fig. 4) present in Tris buffer and are favoured in Na^+ - or K^+ -containing media, respectively (Karlsh *et al.*, 1978). The highest degree of cross-linking to form the 240000- M_r polypeptide and the greatest losses of enzymic activity after cross-linking were associated with these states (Tables 1 and 2). The level of the 240000- M_r polypeptide (Table 1) was not different when Mg^{2+} , K^+ or P_i were substituted for Na^+ . The similar levels of this polypeptide formed in Na^+ - or K^+ -containing media suggest that the F_2 DNS probe did not distinguish between E_1 or E_2 (Fig. 4). However, it should be recognized that even though the presence of a specific ion may favour one state of the enzyme, in most cases several forms exist concurrently. This tends to reduce the possibility of distinguishing one from the other.

In the case of phosphorylated forms of the enzyme, preliminary experiments [the following paper (Harris & Stahl, 1984)] suggested that we could trap the E_1 -P form of the enzyme by using ATP, Na^+ , Mg^{2+} and oligomycin (Fig. 4). Although this is true for short reaction times [the following paper (Harris & Stahl, 1984)], within 1 h much of the ATP is depleted (Fig. 3a), so that in practice a complex mixture of E_1 -P, E_2 -P and non-phosphorylated forms would be expected to exist in various proportions throughout the treatment with F_2 DNS. F_2 DNS is not highly reactive compared with several other bifunctional reagents previously used (Harris & Stahl, 1980), and results obtained (W. E. Harris & W. L. Stahl, unpublished work) suggest that alternative agents may be effective within minutes rather than hours in yielding the maximum amount of cross-linked polypep-

Fig. 4. Simplified reaction scheme and states of the Na^+, K^+ -ATPase

tides. This may be useful in characterizing $\text{E}_1\text{-P}$ and $\text{E}_2\text{-P}$.

The $\text{E}_2\text{-P}$ form is favoured in the presence of Mg^{2+} and P_i , with little contamination by $\text{E}_1\text{-P}$, due to the characteristics of the lamb kidney Na^+, K^+ -ATPase [the following paper (Harris & Stahl, 1984)]. The data suggests that the enzyme in this state differs from non-phosphorylated enzyme (in Tris; Table 3) by having half as much 240000- M_r polypeptide formed. In the case of $\text{E}_1\text{-ATP}$ and $\text{E}_2\text{-P}$, both have similar polypeptide contents, but in the latter lower Na^+, K^+ -ATPase activity was recovered after F_2DNS treatment. Since about the same numbers of thiol groups reacted in each case, different thiol groups must have been exposed to the probe. In this way these three forms of the enzyme may be distinguished.

A most interesting result of the work with F_2DNS is that a new polypeptide with an apparent M_r of 116000 was formed in the presence of ligands favouring a phosphorylated form of the Na^+, K^+ -ATPase. When ouabain is present with Mg^{2+} and P_i , in either the presence or absence of K^+ , high phosphorylation levels of the Na^+, K^+ -ATPase have been found [the following paper (Harris & Stahl, 1984), Table 2], and an $\text{E}_2\text{-P}$ -ouabain intermediate (Siegel *et al.*, 1969) is likely to predominate. The present work shows that, under conditions favouring this intermediate, high levels (22–23% of the total polypeptide) of the 116000- M_r cross-linked product are formed. This is good evidence that $\text{E}_2\text{-P}$ and $\text{E}_2\text{-P}$ -ouabain represent different conformational forms of the enzyme, the latter form having higher thermodynamic stability (Siegel *et al.*, 1969; Schwartz *et al.*, 1975). This offers additional support for the concept that specific ligands involved in Na^+, K^+ -ATPase function modify the tertiary structure of the enzyme.

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