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Biochimica et Biophysica Acta 1235 (1995) 197–204

BBA Biochimica et Biophysica Acta

Phosphorylation/dephosphorylation of reconstituted shark Na^+,K^+-ATPase: one phosphorylation site per $\alpha\beta$ protomer

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Received 12 September 1994; revised 12 December 1994; accepted 10 January 1995

Abstract

In the present investigation reconstitution of Na^+,K^+-ATPase increases the number of phosphorylation sites (EP) of solubilized enzyme from 4.2 ± 0.3 nmol/mg to 6.9 ± 0.6 nmol/mg. The latter figure corresponds to one phosphorylation site per $\alpha\beta$-protomer. A cholesterol content > 10 mol% in the liposome bilayer and a high extracellular $[Na^+]$ are necessary to obtain this high value. Spontaneous dephosphorylation after maximum phosphorylation in Na^+ is biphasic both in solubilized enzyme and after reconstitution. The rate of dephosphorylation compares with the specific hydrolytic Na^+-ATPase activity measured at exactly identical conditions for all three preparations assuming parallel dephosphorylation of at least two phosphointermediates. The distribution of EP-species is found to vary among the three enzyme preparation used, i.e., membrane bound, solubilized, and reconstituted Na^+,K^+-ATPase, however in all the equilibrium is strongly poised away from the E_1P-form.

Keywords: ATPase, Na^+/K^+-; Phosphorylation; Dephosphorylation; Reconstitution; Oligomeric structure

1. Introduction

The oligomeric structure of the Na^+,K^+-ATPase in the native state is still a controversial issue. Several lines of evidence suggest the minimal functional unit to be an oligomer of α,β heterodimers, probably a diprotomeric $(\alpha\beta)_2$-unit. These include radiation inactivation studies [1], ligand binding -and kinetic studies [2,3], and structural studies of two-dimensional crystals [4]. However, equally compelling evidence for an $\alpha\beta$-protomer as the minimal functional unit are present [5–9].

From studies of ligand binding capacity and phosphorylation, it is generally found that the Na^+,K^+-ATPase contains an equal number of sites when measured as phosphorylation capacity, or ouabain- and vanadate binding, or high-affinity ATP binding [10]. However, apparent disagreements exist as to the number of sites per mass of protein: the 2–3 nmol of sites per mg of protein generally found in detergent disrupted membrane preparations can be increased by purification to between 4–6 nmol per mg using detergent solubilization [11–13], or employing zonal centrifugation [14]. Therefore, in both highly purified membrane-bound and in solubilized preparations the maximum phosphorylation corresponds to a site number which is occasionally higher than 1 per $\alpha\beta$-diprotomer and approaches in some preparations 1 per $\alpha\beta$-protomer. Systematic studies of the maximum steady-state phosphorylation following solubilization and reconstitution of Na^+,K^+-ATPase have not previously been performed: very few data for the number of phosphorylation sites of reconstituted Na^+,K^+-ATPase are found in the literature, and usually only relative values are presented [15]. This is often caused by the fact that enzyme orientation after reconstitution is unknown. From one reference [16], however, a low value of about 1.5 nmol per mg of inside-out reconstituted rabbit kidney enzyme can be calculated assuming random insertion of protein in the liposomes, which is usually found in cholate reconstitution. We therefore decided to study the phosphorylation/dephosphorylation reactions using shark Na^+,K^+-ATPase following the steps of solubilization by the non-ionic detergent $C_{12}E_8$ and final reconstitution into liposomes after complete removal of the detergent since in this preparation the orientation of reconstituted Na^+,K^+-ATPase in the liposome population can be accurately determined [17,18].

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SSDI 0005-2736(95)00016-X

2. Methods

The proteoliposomes were produced in 130 mM NaCl, 2 mM $MgCl_2$, and 30 mM histidine, pH 7.0. The optimum hydrolytic activities (V_{max}) in the presence of Na^+ and K^+ (Na^+,K^+-ATPase activity: 3 mM ATP, 130 mM NaCl, 20 mM KCl, and 4 mM $MgCl_2$), or alternatively with only Na^+ (Na^+-ATPase activity: 100 μM ATP, 65 mM NaCl, and 2 mM $MgCl_2$) were estimated from the splitting of [γ-^{32}P]ATP as previously described [19]. For determination of optimum Na^+,K^+-ATPase activity of reconstituted inside-out oriented enzyme 20 mM K^+ was included in the presence of CCCP plus valinomycin to ensure rapid equilibration of K^+ across the proteoliposomes [17].

In the proteoliposomes the hydrolytic activity originates from the turnover of enzyme molecules with exposed ATP-sites, i.e., molecules with their cytoplasmic side facing the outside. These include inside-out, (i/o), oriented enzyme and Na^+,K^+-ATPase reconstituted with both sides exposed (non-oriented enzyme, (n-o)) as opposed to right-side out, (r/o), enzyme, which have their substrate sites shielded inside the liposomes. It should be emphasized that the n-o fraction of Na^+,K^+-ATPase represents reconstituted enzyme in the sense that it is found associated with the lipid phase of the proteoliposomes. It may represent leaky vesicles, or externally adsorbed enzyme [18]. When intended, discrimination between activation of i/o-oriented and n-o enzyme could be achieved by inhibiting exclusively the fraction of n-o enzyme molecules by preincubation of the proteoliposomes with 1 mM ouabain in the presence of 5 mM Mg^{2+} plus 1 mM P_i, followed by a 5 times dilution in the test medium which avoided inhibition of (i/o)-enzyme due to the high MgP_i.

The fractions of (r/o), (i/o), and (n-o)-enzyme for each proteoliposome preparations were determined from functional tests as previously described in details [17,18]: essentially, the fraction of (i/o) is estimated from the fractional activation of hydrolysis by internal (extracellular) K^+ in the presence of external ouabain.

The maximum steady-state level of phosphorylation from ATP of (i/o)-reconstituted Na^+,K^+-ATPase was determined in proteoliposomes produced in 130 mM Na^+, by first inhibiting (n-o)-enzyme with ouabain in the presence of Mg and P_i. To enhance ouabain binding, the external Na^+-concentration was decreased to below 30 mM by centrifugation of the proteoliposomes through Sephadex G-50 columns, as described by Penefsky [20]. The proteoliposomes were then reacted with 10 μM [^{32}P]ATP for 6 s after adjusting the Na^+-concentration to 65 mM. Using this approach the phosphorylation was completely inhibited in unsided preparations, or in proteoliposomes reopened by $C_{12}E_8$ (2 mg/ml), ensuring that only (i/o)-enzyme was phosphorylated in the intact proteoliposomes. Phosphorylation was terminated by adding an acid stop-solution at 0° C containing 10% TCA, 100 mM phosphoric acid, and 20% glycerol. The protein was finally precipitated with 0.15% DOC, followed by washing twice with ice-cold solution containing 0.1% TCA, 10 mM phosphoric acid, and 10 mM sodium pyrophosphate. The protein concentration and radioactivity in the precipitate were determined after resuspension in 1 M NaOH at 55° C. Protein was determined according to Peterson [21]. Calibration curves were recorded by processing in parallel with the assay bovine serum albumin standards including an identical lipid content and composition as in the liposomes. For membrane bound enzyme glycerol and DOC were omitted in the stopping solution.

Phosphorylation from P_i [22] by the 'direct route' was determined in the presence of 3 mM Mg^{2+} and 1 mM P_i in the presence of 0.1 mM digitoxigenin as a substitute for ouabain. The low background was estimated by preincubating with 10 mM CDTA to sequester Mg^{2+} and high Na^+ (130 mM), which is inhibitory. Digitoxigenin was used in place of ouabain since it readily penetrates the

Table 1
Optimal hydrolytic and molecular activities at 10° C and 23° C for different preparations of Na^+,K^+-ATPase measured as Na^+-ATPase or Na^+,K^+-ATPase activity, respectively

		Membranes		Solubilized		Reconstituted	
		Na^+	Na^+,K^+	Na^+	Na^+,K^+	Na^+	Na^+,K^+
Hydrolytic activity							
(μmol/mg per h)	10° C	16.4 ± 0.9 (3)	162 ± 4 (3)	8.4 ± 0.7 (4)	228 ± 16 (3)	22.3 ± 3.3 (3)	§154 ± 3 (3)
	23° C	55.1 ± 1.2 (5)	629 ± 26 (5)	23.7 ± 2.1 (5)	630 ± 23 (4)	92.0 ± 9.7 (3)	§564 ± 21 (4)
Molecular activity							
(min^{-1})	10° C	108 ± 7	1066 ± 62	32.4 ± 1.7	880 ± 46	53.6 ± 1.9	371 ± 10
	23° C	363 ± 21	4143 ± 254	91.7 ± 4.7	2436 ± 90	221 ± 15	1356 ± 42
Phosphorylation sites (nmol/mg)		2.5 ± 0.3 (5)		4.2 ± 0.3 (9)		6.9 ± 0.6 (13)	
Calculated molecular mass (kDa)		395 ± 55		238 ± 17		144 ± 10	

The proteoliposomes were produced in 130 mM NaCl, 2 mM $MgCl_2$, and 30 mM histidine, pH 7.0. In the case of determination of maximum Na^+,K^+-ATPase activity of reconstituted (i/o)-oriented enzyme, 20 mM K^+ was subsequently included in the presence of CCCP plus valinomycin [11] to ensure rapid equilibration of K^+ (§). The maximum steady-state level of phosphorylation was determined as described in Methods. Numbers are means ± S.D. with numbers of observations given in parenthesis.

proteoliposomes supporting phosphorylation of both (i/o)-oriented enzyme and (n-o)-oriented enzyme.

The spontaneous dephosphorylation of (i/o)-oriented Na^+,K^+-ATPase after maximum phosphorylation as described above was followed at either 10° C or 0° C: At different time intervals after chasing of radioactive ATP (10 μM) with cold ATP (1 mM) acid stopping solution was added in the presence of a high Mg^{2+}-concentration (10 mM). ADP- or K^+-supported dephosphorylations were measured by adding 1.5 mM ADP, or 10 mM K^+ with the chasing solution. In the case of proteoliposomes, nigericin (0.5 μg/ml) was included in the K^+-dephosphorylation to rapidly equilibrate the liposomes. In the presence of K^+ the dephosphorylation was measured with a rapid mixing device with varying ageing loops giving reaction times between 15 ms and 1 s.

The distribution of EP-forms in a three-pool model was determined from the fitting of the dephosphorylation data in the presence of ADP or K^+ to exponentials and evaluating the intercepts on the ordinate of the slower phases as described by Nørby et al. [23]. These intercepts represent the ADP-*in*sensitive EP, or K^+-*in*sensitive EP. The K^+-*in*sensitive fraction is identified as E_1P, the K^+-sensitive fraction as $E_2P + E^*P$, whereas the ADP-*in*sensitive fraction is composed of E_2P and the part of E^*P, which is not converted to the directly ADP-sensitive E_1P-form. The latter conversion of E^*P to E_1P is fast enough only at high Na^+ to assume that the ADP-sensitive fraction is the sum of E^*P and E_1P [23]. However, in order to compare qualitative differences between enzyme preparations processed in a similar way, E^*P was simply estimated as the difference between the total EP-content (EP_{tot}) and the sum of the ADP-*in*sensitive and K^+-*in*sensitive fractions. This procedure could conceivably underestimate the E^*P/EP_{tot} ratio [23].

3. Results and discussion

The results given in Table 1 compare the optimum specific hydrolytic Na^+,K^+-ATPase activity and Na^+-ATPase activity (V_{max}) for the three preparations used, i.e., purified membrane-bound enzyme, solubilized enzyme, and reconstituted i/o-oriented enzyme. It is noted that the two activities (Na^+,K^+-ATPase and Na^+-ATPase) do not change proportionally between the three preparations indicating that the degree by which extracellular Na^+ can replace K^+ is different in the preparations. This effect can probably be ascribed to the detergent [12]. Other kinetic differences exist among the preparations as well, as will be described below.

3.1. Phosphorylation

The number of phosphorylation sites increases by solubilization from 2.5 to 4.2 nmol per mg of protein (protein determined by the Lowry method [24] or its modification by Peterson [21]), in accordance with previous results [25], and is probably the result of a purification [14]. In the solubilized Na^+,K^+-ATPase the maximum phosphorylation corresponds to about one phosphorylation site per $(\alpha\beta)_2$-unit, assuming a molecular weight for the α and β polypeptide chains of 112 kDa and 35 kDa, respectively. After reconstitution, by which the detergent is completely removed, phosphorylation by ATP of (i/o)-oriented enzyme gives a maximum steady-state EP-level of 6.9 ± 0.6 ($n = 13$) nmol per mg (i/o)-protein (at 65 mM cytoplasmic Na^+ and 130 mM extracellular Na^+). By phosphorylation via the 'direct route' of reconstituted (i/o + n-o)-enzyme from Mg^{2+} (3 mM) plus P_i (1 mM) in the presence of digitoxigenin and in the absence of Na^+ the phosphorylation level is found to be 6.1 ± 0.2 ($n = 9$) nmol per mg (i/o + n-o)-protein. Both values are close to the theoretical value of 6.8 calculated for 1 site per $\alpha\beta$-unit. The phosphorylation site numbers measured by ATP phosphorylation in reconstituted Na^+,K^+-ATPase are calculated on the basis that only (i/o)-oriented protein is accessible to phosphorylation from external ATP. The experimental conditions assures this since (n-o)-enzyme is inhibited by ouabain (see Methods), and the substrate site of (r/o)-oriented enzyme is directed towards the inside of the liposomes. In contrast, by the P_i-phosphorylation both (i/o) and (n-o)-enzyme have phosphorylation sites exposed and phosphorylation of both orientations are supported by the permeable digitoxigenin. Inclusion of cholesterol in the lipid bilayer of the proteoliposomes in concentrations > 10 mol% is necessary in order to obtain the high phosphorylation site number (see proceeding paper). Readdition of $C_{12}E_8$ to the proteoliposomes in concentra-

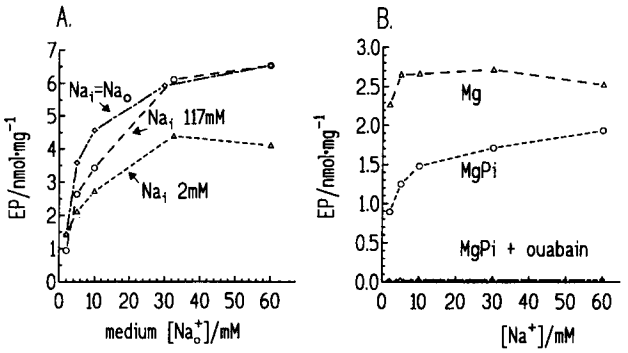

Fig. 1. (A) Maximum phosphorylation level of reconstituted (i/o)-oriented Na^+,K^+-ATPase as a function of the Na^+-concentration in the medium. Phosphorylation was performed for 6 s at 10° C by 10 μM [γ-^{32}P]ATP in the presence of MgP_i ([Mg] = 5 mM, [P_i] = 1 mM) plus ouabain (1 mM). Proteoliposomes contained either 2 mM Na^+ (△), 117 mM Na^+ (○), or an equal Na^+-concentration inside and outside the proteoliposomes (◇). (B) Phosphorylation level as a function of the Na^+-concentration by phosphorylation of membrane-bound enzyme by [γ-^{32}P]ATP (10 μM) in controls ([Mg] = 1 mM) (△), and after preincubation with MgP_i ([Mg] = 5 mM, [P_i] = 1 mM) without (○), or with 1 mM ouabain (◇).

tions that opens the liposomes (2 mg/ml), exposing right-side out reconstituted Na^+,K^+-ATPase, lowers, however, the specific EP-site number to about 4 nmol/mg total protein – the same value as for solubilized enzyme.

The sided action of Na^+ on the maximum steady-state EP-level was investigated for the reconstituted Na^+,K^+-ATPase during conditions where (n-o)-oriented enzyme was inhibited (i.e., in the presence of MgP_i). As seen from Fig. 1A both extracellular and cytoplasmic Na^+ are important, indicating a complex interplay of Na^+ on the phosphorylation reaction at both extracellular and cytoplasmic sites. Optimal conditions to obtain maximum steady-state phosphorylation level apparently require high Na^+-concentrations at both sides. Similar effects have previously been observed by Van der Hijden et al. [16] who showed that a minimum level of either Tris, amine buffers, or Na^+ had to be present at the extracellular side to obtain maximum phosphorylation with cytoplasmic Na^+. The effect could be caused by the same trans-membrane allosteric effect of extracellular Na^+, which is found on cytoplasmic Na^+-activation on both Na^+,K^+-ATPase activity [26] and Na^+-ATPase activity [27].

3.2. Possible artefacts in the determination of EP-site numbers

Since only a small fraction of the total enzyme participate in the phosphorylation reaction after reconstitution, very accurate determinations of enzyme orientation are necessary in order to evaluate both the specific EP-site number and the hydrolytic activity. In the present investigation the fractions of both (i/o)-oriented and (n-o)-enzyme among the different proteoliposome preparations used were very constant: In ten different preparations of proteoliposomes the fractions of (i/o)- and (n-o)-oriented enzyme amounted to 0.128 ± 0.023 and 0.279 ± 0.033 (means $\pm$ S.D., $n = 10$), respectively. It is therefore unlikely that the significant increase in maximum phosphorylation from 4.2 nmol/mg in solubilized preparations to 6.9 nmol/mg following reconstitution is due to an underestimation of (i/o)-oriented enzyme. Moreover, an almost identical EP-site number is obtained in the reconstituted preparation if ATP-phosphorylation is measured without inhibition of the (n-o)-oriented enzyme, i.e., without preincubation in ouabain + MgP_i, and the calculation based on the fractions of (i/o + n-o)-enzyme, lending further confidence to the results. Finally, in dephosphorylation experiments in the presence of K^+ (see below) after phosphorylation of (i/o + n-o)-enzyme a decreased steady-state EP-level corresponding to 13% of total EP was observed by inclusion of nigericin. This figure corresponds exactly to the fraction of (i/o)-oriented enzyme which gets accessible for the fast K^+-dephosphorylation by the addition of nigericin (data not shown).

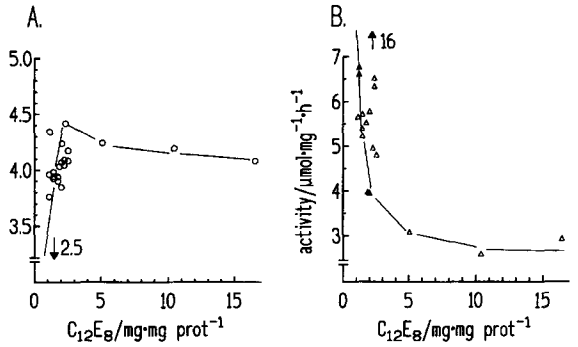

Fig. 2. Detergent effects on maximum phosphorylation level (A) and hydrolytic Na^+-ATPase activity (B) of membrane-bound Na^+,K^+-ATPase at 10° C. (A) The steady-state phosphorylation level (in nmol per mg total protein) was measured with increasing concentrations of $C_{12}E_8$ in the phosphorylation medium. The phosphorylation level first increases and then becomes almost constant in the range of detergent concentrations investigated. In controls, without added $C_{12}E_8$ the phosphorylation level was 2.5 nmol/mg (indicated by the arrow). (B) The curve shows decrease in hydrolytic Na^+-ATPase activity (in μmol per mg total protein per h) measured with increasing $C_{12}E_8$-concentration in the test medium. Without $C_{12}E_8$-addition the Na^+-ATPase activity was 16 μmol/mg per h (indicated by the arrow).

The preincubation of reconstituted Na^+,K^+-ATPase with MgP_i to induce phosphorylation by the direct route and ouabain inhibition of (n-o)-oriented enzyme had no influence on the maximum steady-state EP-level determined by subsequent phosphorylation of (i/o)-oriented enzyme by ATP at 65 mM Na^+. However, for both membrane-bound and solubilized Na^+,K^+-ATPase the EP-level was significant lower ($\sim 40\%$) in the case of MgP_i-phosphorylation prior to ATP-phosphorylation (see Fig. 1B). This indicates that the balance between 'forward' (ATP) and 'backward' (P_i) phosphorylation is different in the three preparations at the ligand conditions used. This competition between forward and backward phosphorylation is dependent on the Na^+-concentration as expected: in membrane-bound (and solubilized) Na^+,K^+-ATPase the Na^+-concentration to obtain optimal steady-state EP-level for the forward phosphorylation is a few mM (Fig. 1B). However, in the presence of MgP_i the optimal Na^+-concentration is considerably increased.

The increase in EP-site number of solubilized enzyme after reconstitution is probably not a result of a further enzyme purification during reconstitution, since the total protein recovery is $> 80\%$, and the patterns in SDS gels are identical for solubilized and reconstituted enzyme (results not shown), nor can it be explained by an underestimation of protein. The latter was carefully investigated recently [28] demonstrating that the Peterson method [21] gave identical or slightly higher values as the original Lowry method and compared if protein was determined from the nitrogen-content. The method is also insensitive to problems with incomplete protein precipitation, since the protein and the EP-content are determined on the same precipitate. Due to the high lipid content of the proteoliposomes it is, however, necessary to include an identical lipid mixture to the bovine serum albumin standards in

order to obtain consistent calibration curves. Since the increase in phosphorylation site number among the three preparations (membrane-bound, solubilized, and reconstituted) is not accompanied by a parallel increase of hydrolytic activity, the molar activities decrease (Table 1). This can probably be ascribed to a detergent interference preferentially with the hydrolytic activity, which is inactivated proportionally more than the maximum phosphorylation level. This is suggested by the data given in Fig. 2 showing that the phosphorylation site number after an initial increase, is only slowly decreasing at increasing detergent concentrations, whereas the hydrolytic Na^+-ATPase activity rapidly decreases even at very low detergent concentrations.

The possibility that reconstitution induces a second phosphorylation site on the enzyme, comparable to the low-affinity superphosphorylation previously reported [29], is considered unlikely with the present preparation since maximum phosphorylation was essentially identical for low ATP (10–25 μM) and high ATP (1000 μM) in both membrane-bound enzyme and reconstituted enzyme at 0° C, without indication of an overshoot phenomenon (see Fig. 3A,B).

3.3. Dephosphorylation

In Table 2 data from spontaneous dephosphorylations at 10° C of the three different enzyme preparations are compared (membrane-bound, solubilized and reconstituted). As shown, each preparation has a discrete dephosphorylation characteristic, indicating distinct decay kinetics and interconversion rates between the EP-species in the EP-pool. The data for spontaneous dephosphorylation for solubilized and reconstituted enzyme clearly deviated from monoexponential decay and contained as a minimum both a rapid and a slow component (indicated by the exponential slopes, λ), whereas for membrane bound enzyme monoexponential and biexponential fit were equally good as judged by *F*-tests, at least at 10° C. However, at 0° C also membrane-bound enzyme shows biexponential dephosphorylation (Fig. 4).

In order to evaluate the distribution of EP-species, results are given in Fig. 4, which show spontaneous dephosphorylation at 0° C of membrane-bound, solubilized, and (i/o)-reconstituted Na^+,K^+-ATPase, together with ADP- or K^+-activated dephosphorylation. The low temperature is necessary in order to follow the K^+-activated dephosphorylations. ADP did not appreciable accelerate dephosphorylation in membrane bound and solubilized preparations compared to the spontaneous dephosphorylation (Fig. 4A,B) indicating modest fractions of E_1P, whereas in reconstituted vesicles a small acceleration is apparent (Fig. 4C). The K^+-acceleration of dephosphorylation was less pronounced in reconstituted vesicles than in membrane-bound and solubilized preparations. Especially, the initial very fast phase is absent in reconstituted preparations. This is almost certainly due to a restricted K^+-permeability even in the presence of nigericin. Calculations using the 3-pool EP-models of Nørby et al. [23], Yoda and

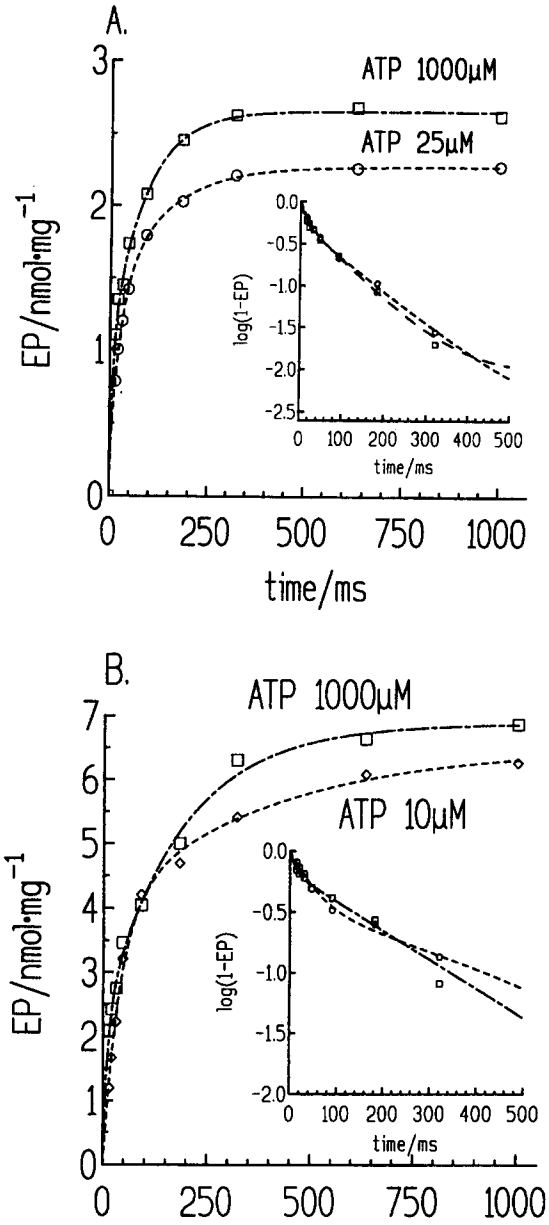

Fig. 3. Phosphorylation at 0° C of (A) membrane-bound Na^+,K^+-ATPase at 25 μM (○) and 1000 μM (□) ATP, and (B) reconstituted Na^+,K^+-ATPase at 10 μM (◇ (inset ○)) and 1000 μM (□) ATP. The curves are computer-fit to the data using the biexponential association equation $EP = f_1(1-\exp(-\lambda_1 t)) + f_2(1-\exp(-\lambda_2 t)) + EP_0$ with the following exponential slopes (λ) and fractions (f): In the case of membrane-bound enzyme (A) at 25 μM ATP, $\lambda_1 = 58 \pm 8$ s^{-1}, $\lambda_2 = 9.2 \pm 1.2$ s^{-1}, $f_1 = 1.10 \pm 0.12$ nmol/mg, and $f_2 = 1.16 \pm 0.12$ nmol/mg. In the same preparation at 1000 μM ATP: $\lambda_1 = 123 \pm 32$ s^{-1}, $\lambda_2 = 11.4 \pm 1.3$ s^{-1}, $f_1 = 1.03 \pm 0.12$ nmol/mg, and $f_2 = 1.62 \pm 0.11$ nmol/mg. For reconstituted enzyme (B) at 10 μM the fitting coefficients were: $\lambda_1 = 25 \pm 4$ s^{-1}, $\lambda_2 = 2.6 \pm 1.5$ s^{-1}, $f_1 = 4.0 \pm 0.6$ nmol/mg, and $f_2 = 2.5 \pm 0.4$ nmol/mg. In the same preparation at 1000 μM ATP: $\lambda_1 = 91 \pm 28$ s^{-1}, $\lambda_2 = 5.4 \pm 0.8$ s^{-1}, $f_1 = 2.3 \pm 0.3$ nmol/mg, and $f_2 = 4.6 \pm 0.3$ nmol/mg. The insets show the same data and curves depicted in a semi-logarithmic plot given as residual relative EP, (1 − EP), vs. time.

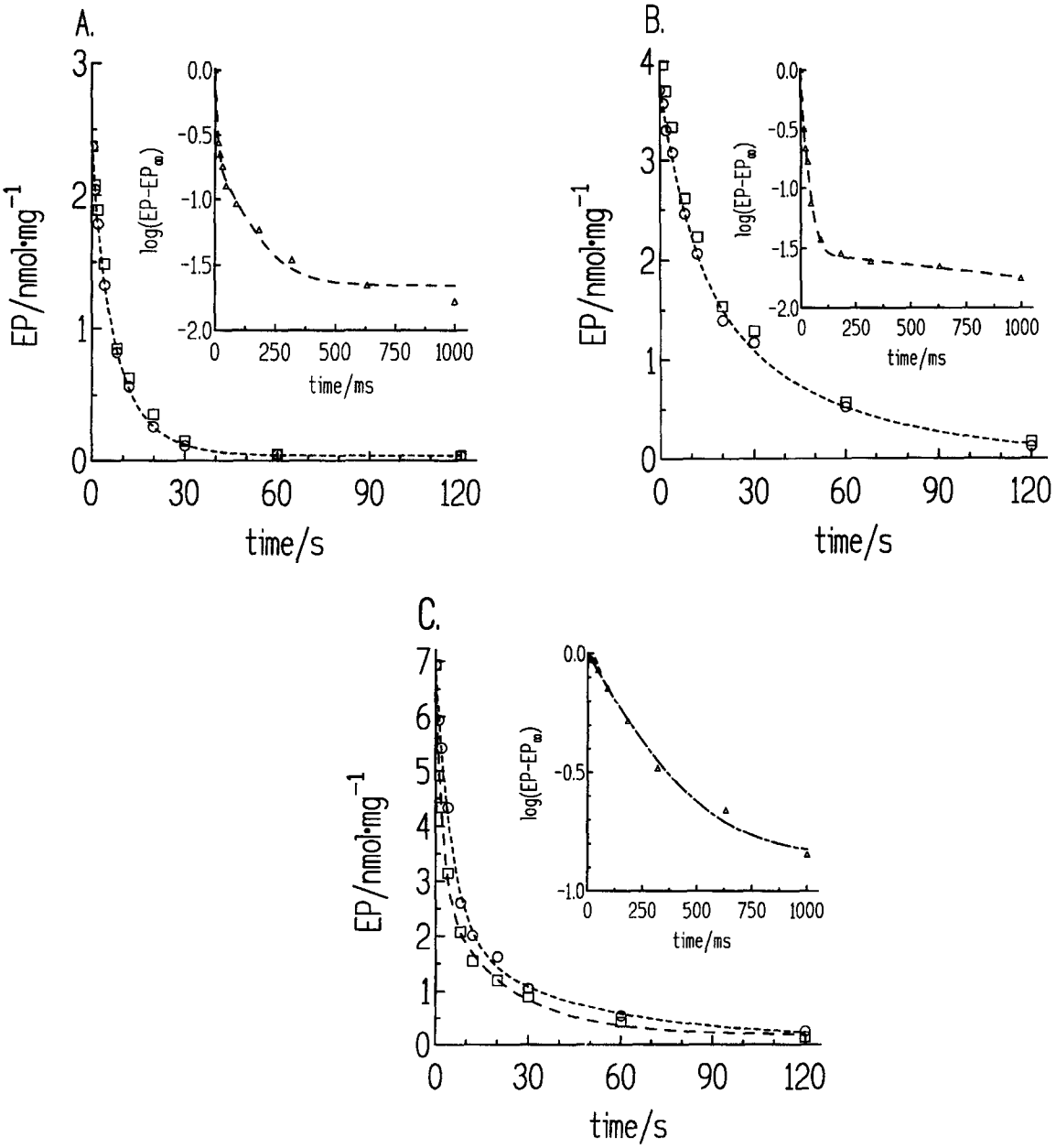

Fig. 4. Dephosphorylation of (A) membrane bound, (B) solubilized, and (C) reconstituted (i/o)-oriented Na^+,K^+-ATPase at 0° C. The time-course of spontaneous dephosphorylation (○) was followed by adding stopping solution at indicated times after chasing of radioactive ATP (10 μM) with cold ATP (1 mM) in the presence of high Mg^{2+} concentration (10 mM). ADP-supported dephosphorylation (◇) was measured by adding 1.5 mM ADP with the chase solution. K^+-supported dephosphorylation (△) was measured by the addition of 10 mM K^+. In the case of proteoliposomes nigericin (0.5 μg/ml) was included in the K^+-dephosphorylation to rapidly equilibrate the liposomes. The K^+-dephosphorylations were completed within 1 s, except for reconstituted enzyme, and were measured using a rapid mixing device. These K^+-dephosphorylations are shown in the insets as relative values on a log scale during the initial first second. All shown curves are computer-fits to the data using exponential decay, $EP = f_1 \cdot \exp(-\lambda_1 t) + f_2 \cdot \exp(-\lambda_2 t) + EP_\infty$, with the following exponential slopes (λ) and fractions (f): Spontaneous dephosphorylation of membrane-bound enzyme (A): $\lambda_1 = 0.17\ s^{-1}$, $\lambda_2 = 0.05\ s^{-1}$, $f_1 = 2.16$ nmol/mg, $f_2 = 0.72$ nmol/mg. Solubilized enzyme (B): $\lambda_1 = 0.11\ s^{-1}$, $\lambda_2 = 0.03\ s^{-1}$, $f_1 = 1.70$ nmol/mg, $f_2 = 2.35$ nmol/mg. Reconstituted enzyme (C): $\lambda_1 = 0.19\ s^{-1}$, $\lambda_2 = 0.02\ s^{-1}$, $f_1 = 4.86$ nmol/mg, and $f_2 = 1.94$ nmol/mg. For membrane-bound and solubilized enzyme the time-course of dephosphorylation was independent of ADP, whereas for reconstituted enzyme ADP accelerated dephosphorylation giving: $\lambda_1 = 0.43\ s^{-1}$, $\lambda_2 = 0.04\ s^{-1}$, $f_1 = 4.11$ nmol/mg, $f_2 = 2.51$ nmol/mg. The curves for K^+-dephosphorylations during the initial 1 sec, shown in the insets on a log scale, were better fitted to the sum of three exponentials in the case of membrane-bound enzyme ($\lambda_1 = 170\ s^{-1}$, $\lambda_2 = 23\ s^{-1}$, $\lambda_3 = 1.8\ s^{-1}$, $f_1 = 1.7$ nmol/mg, $f_2 = 0.5$ nmol/mg, and $f_3 = 0.2$ nmol/mg) and for solubilized enzyme ($\lambda_1 = 175\ s^{-1}$, $\lambda_2 = 45\ s^{-1}$, $\lambda_3 = 0.5\ s^{-1}$, $f_1 = 1.9$ nmol/mg, $f_2 = 1.9$ nmol/mg, and $f_3 = 0.12$ nmol/mg). For reconstituted enzyme the initial very fast phase was absent and the sum of only two exponentials described the data ($\lambda_1 = 4.4\ s^{-1}$, $\lambda_2 = 0.02\ s^{-1}$, $f_1 = 6.17$ nmol/mg, $f_2 = 1.02$ nmol/mg). The distribution of EP-forms in the three different preparations calculated from the exponential computerfits to the ADP- and K^+-chase data, as explained in Methods, is (%): (A) $E_1P/E^*P/E_2P$ = 6:19:75, (B) $E_1P/E^*P/E_2P$ = 3:46:51, and (C) $E_1P/E^*P/E_2P$ = 14:48:38.

Yoda [15], and Lee and Fortes [30] show that the EP-distribution is comparable in membrane-bound, solubilized, and reconstituted enzyme in being poised towards E^*P and E_2P, but the E^*P proportion increases at the expense of E_2P in the solubilized and reconstituted enzyme preparations (see Fig. 4). These small variations could reflect effects of either the detergent, or differences in cholesterol content in the membranes and liposome bilayer, on the one hand, and the lipid/protein/detergent mixed micelles, on the other hand. Cholesterol has previously been demonstrated to influence the EP-distribution in proteoliposomes [31], however the method employed there only uses one point in the dephosphorylation curves (1 s) which in the present study would underestimate the fraction of E^*P.

Essentially, the 3-pool model contains three phosphointermediates E_1P, E^*P, and E_2P, two of which dephosphorylates rapidly in the presence of K^+ (i.e., the directly K^+-sensitive E_2P and E^*P through rapid conversion to E_2P), and one (E_1P) in the presence of ADP:

$$E_2P \rightleftharpoons E^*P \rightleftharpoons E_1P$$

$$\downarrow +K^+ \downarrow \qquad \downarrow +ADP$$

A detailed analysis of the dephosphorylation kinetics using the 3-pool model as a frame of reference has not been attempted, although this will become feasible as more data will be accumulated. However, certain common trends can be demonstrated: (i) for all three preparations the sum of ADP-sensitive EP and K^+-sensitive EP exceeds 100%, indicating at least three EP-species, (ii) the distribution of EP-species varies among the three preparations, but the distribution is in all cases poised to the $E^*P + E_2P$, (iii) for all three enzyme preparations the rate of dephosphorylation, taking into account both the fast and the slower component, is sufficient to account for the hydrolytic Na^+-ATPase activities measured at exactly comparable conditions (which are sub-optimal), as expected if the phosphoenzymes are assumed consecutive intermediates in a single reaction pathway, with at least two of them dephosphorylating with different rate constants. This is shown in Table 2, where the hydrolytic Na^+-ATPase activity is compared to the rate of dephosphorylations. The latter is given by $d(EP - EP_\infty)/dt = \lambda_1 f_1 + \lambda_2 f_2$, in which f is the fraction with indicated exponential slope λ. However, for all three preparations a well-known apparent controversy exist for dephosphorylation in the presence of K^+: whereas the initial rate exceeds by far the Na^+,K^+-ATPase activity, the turnover calculated as the E_1P-fraction multiplied by the exponential slope of the slow component in the K^+-dephosphorylation after phosphorylation in pure Na^+ is too low to accommodate the overall turnover during Na^+/K^+-exchange measured at exactly comparable conditions. This has previously been demonstrated also for membrane-bound Na^+,K^+-ATPase (for references, see [32]). The controversy rests of course on the assumption that the exponential slope after complete K^+-induced dephosphorylation of E_2P and E^*P represents or exceeds the steady-state forward rate constant for conversion of E_1P. Moreover, proper resolution of exponentials in the fitting to the K^+-dephosphorylation data must be presumed. As indicated in Fig. 4 up to three exponentials are apparently needed in order to simulate the K^+-chase experiments at 0° C. However, even disregarding the slowest very small component would not remove this obstacle and increase the calculated rate $E_1P\lambda_2$ to a value compatible with the overall turnover during Na^+/K^+-exchange.

The observations reported in this paper show that reconstitution of solubilized Na^+,K^+-ATPase from shark into lipid vesicles increases the number of phosphorylation sites per mg (i/o)-protein from about one per $(\alpha\beta)_2$-unit to one per $\alpha\beta$-protomer. Concomitantly, the molar activity decreases proportionally. Similar high binding capacities using ouabain or vanadate have been approached previously in membrane-bound enzyme preparations only by collecting the fractions after zonal centrifugation with peak Na^+,K^+-ATPase activity [14]. The results are most readily explained from a relieved detergent interference by reconstitution with the measurements of phosphorylation level. Apparently, catalysis is more detergent sensitive than preservation of phosphorylation sites. This is in accord with the observation that readdition of detergent to the reconsti-

Table 2
Spontaneous dephosphorylation at 10° C for membrane-bound, solubilized, and reconstituted Na^+,K^+-ATPase

	λ_1 (s^{-1})	λ_2 (s^{-1})	f_1 (nmol/mg)	f_2 (nmol/mg)	dEP/dt (μmol/mg per h)	
					calculated	measured
Membrane	0.43 ± 0.04	–	2.84 ± 0.04	–	4.40 ± 0.35	4.90 ± 0.41
Solubilized	1.19 ± 0.45	0.128 ± 0.009	0.94 ± 0.14	2.95 ± 0.15	4.91 ± 1.5	5.61 ± 0.49
Reconstituted	0.61 ± 0.16	0.048 ± 0.018	4.97 ± 0.84	1.93 ± 0.67	11.2 ± 1.3	11.4 ± 0.68

The maximum steady-state level of phosphorylation of i:o reconstituted Na^+,K^+-ATPase was determined in proteoliposomes produced in 130 mM Na^+ by inhibiting n-o oriented enzyme with ouabain. The rate constants, λ, and fractional EP-site number, f, were estimated by fitting exponential functions to the data. The initial rate of dephosphorylation, assuming parallel dephosphorylation, was calculated according to $d(EP - EP_{tot})/dt = \lambda_1 \cdot f_1 + \lambda_2 \cdot f_2$. The measured specific hydrolytic Na^+-ATPase activity was assessed under exactly identical conditions as used for dephosphorylation, i.e., 1 mM ATP and 10 mM Mg^{2+} (see legend to Fig. 1). All figures are means ± S.D. ($n = 3$).

tuted Na^+,K^+-ATPase also reestablishes the lower site number of about 4 nmol/mg, as found in solubilized enzyme, and as previously measured in reopened proteoliposomes using vanadate [17]. The complex biphasic relation between detergent and maximum phosphorylation seen in Fig. 2A does not exclude that a maximum phosphorylation corresponding to the theoretically 6.8 nmol/mg could be obtained if a proceeding progressive detergent inactivation could be relieved. Another possibility is that the lower site number in solubilized enzyme as compared to reconstituted enzyme could be due to the presence of inactive enzyme, or contaminating protein which is not reconstituted into the liposomes. However, the high recovery of total protein after reconstitution speaks against this possibility. Alternative explanations considered less likely could be, that in the membranes the minimal functional units are diprotomeric $(\alpha\beta)_2$-units with negative cooperativity between protomers, so that phosphorylation of one protomer alone is sufficient to support the turnover of the diprotomer. By solubilization and reconstitution monomerization results to various extent whereby each protomer must be phosphorylated in order to turn over. However, since increasing $C_{12}E_8$ concentration does not induce the high EP-site number, interference in the measurements of EP-sites from detergents must be assumed in addition. The conflicting reports in the literature whether or not detergent monomerization increases the phosphorylation site number [13,25] could be due to such variable interference from the necessary high concentration of detergent employed. A recent report [29] showing initial phosphorylation levels corresponding to two sites per ouabain site at high ATP concentrations in pig kidney Na^+,K^+-ATPase could not be confirmed in the present investigation using either membrane-bound or reconstituted shark enzyme: the maximum level of phosphorylation as well as the shape of the phosphorylation curve were very similar at 0° C for low and high ATP, except for an increased initial rate at the higher ATP-concentration (Fig. 3).

Acknowledgements

I thank Drs. J.C. Skou and J.G. Nørby for useful discussions. Hanne Zakarias is gratefully acknowledged for excellent technical assistance. This work was supported by grants from the Danish Medical Research Council, The Novo Foundation, and The Danish Biomembrane Research Centre.

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