

Phosphorylation by Inorganic Phosphate of Sarcoplasmic Membranes

Bernhard Rauch, Dorothee v. Chak, and Wilhelm Hasselbach

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

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The calcium transport protein of the sarcoplasmic reticulum accepts inorganic phosphate rapidly when phosphorylation is initiated either by the addition of phosphate or magnesium ions to the calcium free protein. Phosphorylation proceeds much more slowly when it is initiated by the addition of the calcium chelator ethyleneglycol-bis(β -aminoethyl ether)-N,N'-tetraacetic acid (EGTA) to the phosphate and magnesium containing assay. The time course of phosphorylation following immediately calcium removal is monophasic at all temperatures between 20° and 37 °C. In contrast, phosphorylation of the calcium free enzyme becomes biphasic at temperatures above 25 °C. The biphasic time course does not only apply to net formation of phosphoprotein but also to its exchange with medium phosphate. On addition of calcium, the phosphoprotein decays in a biphasic process the time constants of which are much longer than those observed for phosphoprotein formation. The temperature dependence of the rate as well as of the extent of phosphoprotein formation indicate a discontinuity in the reactivity of the protein.

Introduction

Considerable uncertainty has arisen lately, concerning the existence of different phosphorylated intermediates which are formed in the sarcoplasmic membranes by the incorporation of inorganic phosphate. Makinose¹ and Yamada *et al.*² observed that calcium loaded vesicles incorporated 1–2 nmol phosphate when they were exposed to calcium free media at pH 7.0. Since only small amounts of phosphate (0.1–0.2 nmol/mg) were incorporated in the absence of a calcium gradient into the membranes of unloaded vesicles, it was assumed that phosphate incorporation was causally linked to the existence of a calcium gradient across the membranes. The phosphoryl group of the intermediate formed in the presence of a calcium gradient could rapidly be transferred to ADP whereby the level of phosphoprotein dropped to low values. The contribution of the calcium gradient to phosphoprotein formation, however, became uncertain when Kanazawa and Boyer³, and Masuda and de Meis⁴ demonstrated that the membrane could accept phosphate even in the absence of a calcium gradient, and good yields were obtained when the temperature of the assays was elevated and the pH of the media was reduced

from 7.0 to 6.0. In the following, the relation between the intermediates formed in the absence and presence of a calcium gradient remained open. The fact that the phosphorylated intermediates after fixation by acification could not be distinguished anymore is a main handicap. The most promising criterion by which the intermediates in the native protein can be differentiated are their kinetic and thermodynamic properties. Beil and Hasselbach⁵ have demonstrated that conditions can be found under which practically only gradient dependent phosphoprotein is formed. It is characterized by its slow rate of formation, its low heat of formation, and its sensitivity to the calcium ionophore X 537 A as well as to ADP. In these studies some complementary observations concerning the kinetic and the thermodynamic properties of the gradient independent phosphoprotein will be given.

Materials and Methods

Preparation of sarcoplasmic reticulum vesicles

The vesicles of sarcoplasmic reticulum were prepared from rabbit skeletal muscles as described by Hasselbach and Makinose⁶.

Rapid quenching apparatus

To measure the kinetic of E–P formation, a modified quench flow apparatus as described by Kröger and Klingenberg⁷ was used.

The reaction time was varied between 22 milliseconds and 3 seconds by changing the rate of flow

Requests for reprints should be sent to Prof. Dr. Wilhelm Hasselbach, Max-Planck-Institut für medizinische Forschung, Abteilung Physiologie, Jahnstr. 29, D-6900 Heidelberg.

Abbreviation: EGTA, ethyleneglycol-bis(β -aminoethyl ether)-N,N'-tetraacetic acid.



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in the reaction chamber and by using reaction chambers of variable size.

All the tubes, the reservoir barrels, the mixing chambers, and the reaction chamber were bored into a block of stainless steel. The drive pistons and the valve for refilling the syringes were made of teflon. In order to avoid fluid leakage, a fluid tight seal was made by means of rubber O-rings set into grooves around the circumference of each teflon piston.

The reservoir syringes and the reaction chamber were thermostated, and temperature was controlled by a thermocouple able to registrate changes in temperature in the range of 0.1 °C. A valve changing automatically between waste- and assay-position when the drive pistons move down assures that the remaining solution in the tubings from the previous drive stroke is not added to the new sample. Furthermore, this system has the advantage that measurement takes place before the flow has reached a steady rate. The performance of the apparatus was tested by studying the kinetics of the hydrolysis of 0.5 mM 2,4 dinitrophenyl acetate with 0.25 M potassium hydroxide at 20 °C. From the slope of the first-order-plot the pseudo-first-order rate constant is calculated at 14.5 sec yielding a second order rate constant of 58 M⁻¹sec⁻¹ which is in good agreement with the values reported in literature^{8,9}.

Assays

If not described otherwise, the substrate syringe contained 5 mM (³²P) P_i, 5 mM EGTA, 10 mM Tris-maleate pH 6.0 and 20 mM MgCl₂. The enzyme syringe had all the components of the substrate syringe minus MgCl₂ and contained microsomal protein at a concentration of 1.0 mg/ml. The temperature was adjusted to 30 °C. The reaction was stopped with icecold 10% trichloroacetic acid.

After separating the ³²P-labeled protein from the supernatant by centrifugation it was resuspended in a 10% trichloroacetic acid solution containing 50 mM phosphoric acid. The resuspended protein of each sample was collected on glass fiber and washed with at least 100 ml of the same solution. The protein pellet on the filter was suspended in 3 ml 0.01 M NaOH, heated in boiling water for 3 min. Subsequently 1 ml 0.1 M H₃PO₄ was added. After cooling an aliquot was taken for counting in a liquid scintillation counter.

Results

In the presence of a calcium gradient the incorporation of phosphate proceeds slowly at reduced temperature so that the time course of the reaction can easily be followed by conventional

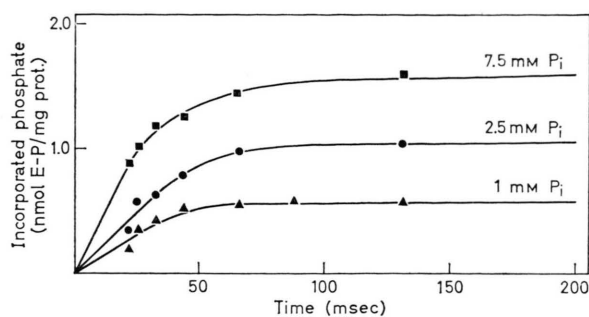
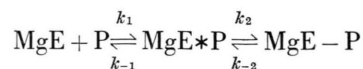
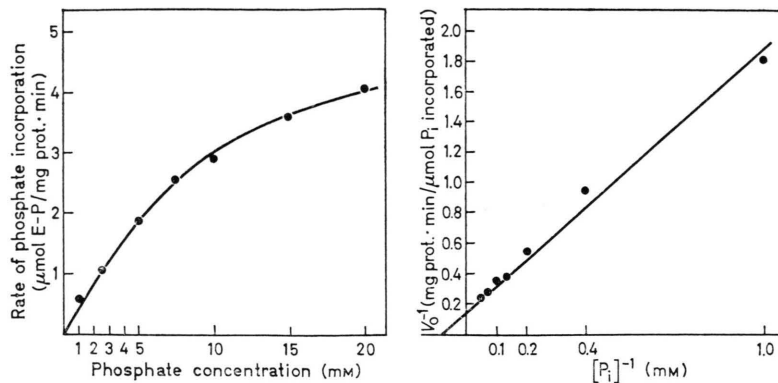


Fig. 1. Time course of phosphoprotein formation at three different phosphate concentrations (▲ 1 mM, ● 2.5 mM, ■ 7.5 mM). The other conditions were as described in materials and methods.

methods. In contrast, in the absence of a calcium gradient phosphoprotein is rapidly formed and exchanges rapidly with phosphate in the medium requiring a rapid mixing technique to resolve its kinetics. When vesicular suspension containing 5 mM EGTA and 1–20 mM phosphate are supplemented with magnesium ions, 10 mM, phosphoprotein is formed, and vice versa, phosphoprotein rises rapidly when the vesicles suspended in EGTA and magnesium containing media are supplemented with phosphate. Fig. 1 demonstrates the time course of phosphoprotein formation at three different phosphate concentrations at 30 °C and pH 6.0. The apparent second order rate constant associated with phosphoprotein formation is in the range of 900 M⁻¹sec⁻¹. Fig. 2a in which the initial rates for phosphoprotein formation are plotted against the phosphate concentrations shows that the rate does not increase linearly. The same nonlinearity is observed for the dependence of the rate of phosphoprotein formation on the concentration of magnesium. These findings exclude a one step phosphorylation mechanism irrespective of the initiation of phosphorylation by magnesium or phosphate and suggest the following two step reaction as the simplest possible sequence with MgE*P as an acid labile intermediate.



Under the assumption that the bimolecular process occurs much faster than the transition of the intermediate complex –MgE*P– to the final product the following relationship between the reciprocal value of the initial rate and the concentration of phosphate is obtained.



Figs 2 a, 2 b. Dependence of the initial rate of phosphoprotein formation on phosphate concentration. Besides the variation of phosphate concentration the reaction conditions were as described in materials and methods.

$$\frac{1}{V} = \frac{1}{k_2} + \left(\frac{1}{K \cdot k_2} \right) \cdot \frac{1}{P} \quad K = k_1 / (k_{-1} + k_2) \sim k_1 / k_{-1} \quad (1)$$

Fig. 2 b illustrates this linear relationship for the observed in initial rates.

The value for the maximum rate obtained from the intercept on the ordinate gives $6.6 \mu\text{mol mg}^{-1} \cdot \text{min}^{-1}$. From the intercept and the slope of the straight line of the graph the apparent affinity constant of the enzyme for phosphate of $K = 90 \text{ M}^{-1}$ is obtained. As to be expected from the low affinity of the membrane for phosphate the steady state level of phosphoprotein increases in the range of the applied concentration for phosphate. Extrapolation of the steady state value for phosphorylation by double reciprocal plotting gives maximum phosphorylation levels of 3 nmol/mg and an apparent affinity constant of $K' = 570 \text{ M}^{-1}$ (Fig. 3). The difference between this figure and the figure obtained from kinetic measurements reflects the dependence of the steady state level of phosphoprotein on k_2 as well as on k_{-2} [Eqn (2)] and can be used to compute the latter constant.

$$\frac{\text{MgE}_0}{\text{MgE} - \text{P}} = \frac{k_2 + k_{-2}}{k_2} + \frac{k_{-2}}{k_2 \cdot K} \cdot \frac{1}{P} \quad (2)$$

From this equation the following relationship for K' is obtained

$$K' = K \left(1 + \frac{k_2}{k_{-2}} \right)$$

which in combination with Eqn (1) gives a value of $1.2 \mu\text{mol mg}^{-1} \cdot \text{min}^{-1}$ for the rate with which the phosphoprotein decays in the reverse reaction. Assuming 3 nmol/ml as the amount of enzyme in the preparation, 36 sec^{-1} and 6.6 sec^{-1} are obtained

as tentative rate constants for k_2 and k_{-2} , respectively, at 30°C .

The steady state level for phosphoprotein increases considerably when the temperature is raised corresponding to an enthalpy of formation of $12\,000 \text{ cal/mol}$ (Fig. 4) ^{5, 10}. This is the reason for measuring the amount of phosphoprotein at elevated temperature. Since with rising temperature the rate of phosphate incorporation increases considerably, reliable measurements become more difficult to perform. From the data of Fig. 4 an activation enthalpy for phosphoprotein formation of 110 kJ ($26\,300 \text{ cal/mol}$) is obtained in the temperature range of $15 - 25^\circ \text{C}$ and a lower activation enthalpy of 74 kJ ($17\,700 \text{ cal/mol}$) between 25 and 35°C . Due to the high temperature coefficient already small inaccuracies in measuring the temperature can give rise to considerable rate changes. The

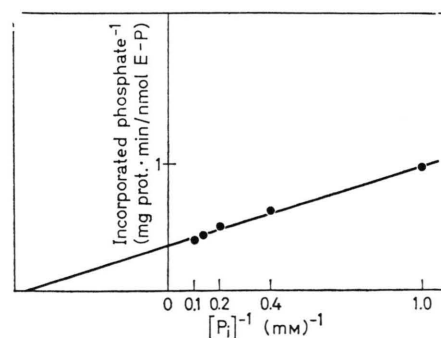


Fig. 3. Double reciprocal plot of the steady state level of phosphoprotein versus the concentration of added phosphate. Phosphorylation was measured at pH 6.0 and 30°C 1 min after starting the reaction with 10 mM MgCl_2 (final concentration). The reaction media contained phosphate as indicated in the graph, 5 mM EGTA , $10 \text{ mM Tris maleate}$ and $0.5 \text{ mg/ml vesicle protein}$.

Table I. Initial rates and half times of phosphoprotein formation.

	Concentration of P _i in reaction media [mM]	Temperature [°C]	Initial rates [μ mol E-P/mg prot. min]	Half time:		Fast phase of phosphoprotein formation [%]
				fast phase	slow phase	
				[msec]		
Net formation of phosphoprotein (calcium free medium)	5	20	0.7–0.9		240–330	
	12		1.5–1.7		90–100	
	5	30	1.8–2.2	16–20	90–150	~55
	12			9–11	70–85	~70
Net formation of phosphoprotein (reaction started by calcium removal)	5	20	0.3–0.4		350–440	
	5	30			80–96	
Turnover	5	20	0.2–0.3		310–480	
	12		0.4–0.5		250–310	
	5	30	0.8–1.2	14–15	120–170	25
	12		1.5–1.8	10–11	80–90	30
Turnover (sonicated vesicles)	5	20	1.1–1.2	17–18	140–150	60
	12		1.7–1.9	14–15	120–130	50
Decay of phosphoprotein induced by calcium addition	5	30		120–140	1400–1600	44

The reaction conditions for phosphoprotein net formation in calcium free medium and net formation of phosphoprotein started by calcium removal were as described in materials and methods and in Fig. 5, respectively. To measure the turnover rates, a suspension containing 1.0 mg/ml of microsomal protein, 5 mM P_i, 5 mM EGTA, 10 mM Tris maleate and 10 mM MgCl₂ at pH 6.0 was mixed with an equal volume of reaction medium without vesicle protein but with ³²P_i at the same concentration. For measuring the decay of phosphoprotein induced by calcium, reaction conditions were used as described in Fig. 6.

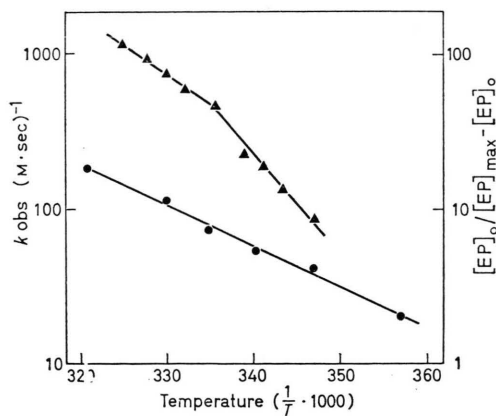


Fig. 4. Temperature dependence of the apparent second order rate constant (▲) and of the level of phosphoprotein formed in the absence of a calcium gradient (●). For measuring the rate constants reaction conditions were used as described in materials and methods. Phosphoprotein level was measured with 30 mM P_i, 10 mM MgCl₂, 10 mM EGTA, 0.4 mg/ml vesicles at pH 6.0. The reaction was quenched after 1 min when E-P formation had reached an optimal value.

described rapid formation of phosphoprotein is the characteristic property of the calcium free enzyme.

A conclusive kinetic analysis is made difficult because at 30 °C net phosphorylation as well as the phosphate exchange exhibit two-phasic time courses. The amplitudes of the two phases and the corresponding half times of 15–20 msec for the rapid and 80–140 msec for the slow phase are collected in Table I. A slow diffusion of phosphate across the sarcoplasmic membrane to reactive sites at the internal surface of the vesicles could largely be excluded as a possible explanation for this slow phase of phosphate incorporation: Intense sonication only leads to partial reduction of the slow phase which is connected with a significant lower yield of phosphoprotein. Yet, the biphasic time course of phosphorylation nearly disappears when the temperature is reduced below 25 °C and only the phase for phosphoprotein net formation as well as for phosphate exchange remains observable. The fast initial phase disappears likewise nearly completely at all temperatures when phosphorylation is initiated not by the addition of magnesium or phosphate but by the removal of calcium from the protein. The rate is compatible with that of the slow phase observed when the calcium free enzyme is phosphorylated

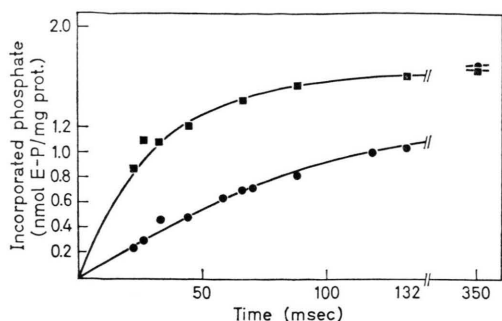


Fig. 5. Time course of phosphoprotein net formation started with MgCl_2 and EGTA (●) in comparison with phosphoprotein net formation in calcium free medium (■). In the first case, the substrate syringe contained 5 mM P_i , 10 mM Tris maleate, 10 mM EGTA, and 20 mM MgCl_2 . The enzyme syringe only contained 5 mM P_i , 10 mM Tris maleate, and 1.0 mg/ml vesicle protein. The phosphoprotein net formation in calcium free medium was performed as indicated in materials and methods. Both reactions took place at 30 °C and pH 6.0.

(Table I and Fig. 5). Obviously, the removal of calcium itself or a subsequent reaction step occur relatively slowly and determine the rate of phosphate incorporation. The data in Table I show that not only the half time of net formation but also that of phosphate exchange depend on the concentration of phosphate. This latter dependence is a finding which is not consistent with the tentative reaction sequence.

The two rate constants observed at 30 °C suggest that at higher temperatures two classes of phosphoenzymes might exist. This notion is supported by the twophasic time course of the decay of phosphoprotein induced by the addition of calcium (Fig. 6). A rapid phosphate liberation of approximately 50%

of the incorporated amount is followed by a much slower proceeding phosphate release. Half times of ~ 130 msec and ~ 1.6 sec characterize fast and slow decay of phosphoprotein, respectively.

Conclusions

Continuous ATP synthesis during calcium release from calcium loaded vesicles requires continuous removal of calcium from external binding sites of the vesicles¹¹. As long as these sites are occupied, neither calcium is released nor phosphate is incorporated into the transport protein, nor ATP synthesis occurs, although a considerable calcium gradient can exist. The removal of four calcium ions from the transport protein is necessary to incorporate one phosphate residue. The initial rates of phosphate incorporation at 20 and 30 °C following immediately the addition of EGTA were found to be $0.3 \mu\text{mol}/\text{min} \cdot \text{mg}$ protein and $0.7 \mu\text{mol}/\text{min} \cdot \text{mg}$ protein, respectively. Assuming that one mg of protein corresponds to 3 nmol of phosphoacceptor sites, rate constants of 1.7 sec^{-1} and 3.9 sec^{-1} for phosphate incorporation result. For this slowly proceeding phosphate incorporation following calcium removal, two explanations may be suggested.

1. The phosphate accepting protein configuration is slowly formed.
2. The phosphate accepting configuration of the protein might be formed rapidly, but phosphate incorporation itself is the rate determining step.

In the first case, net phosphorylation of the calcium free protein after having been incubated for some minutes in EGTA could be phosphorylated

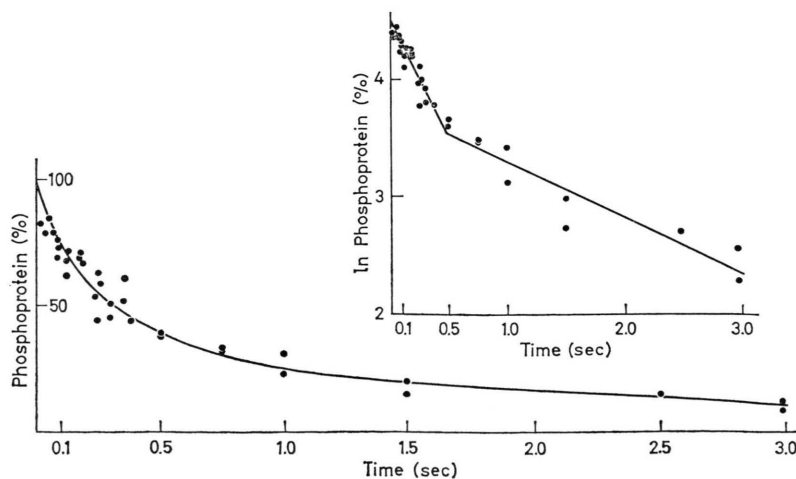


Fig. 6. Time course of the decay of phosphoprotein induced by the addition of calcium. The maximum amount of labelled E-P was measured 1 min after incubation of 1.0 mg/ml sarcoplasmic vesicles in a reaction mixture, containing 10 mM Tris maleate, 5 mM EGTA, 5.5 mM Na-glycerophosphate, 10 mM MgCl_2 , and 5 mM P_i at pH 6.0 and 30 °C. To measure the phosphoprotein decay by addition of calcium, 1.3 ml of the same reaction mixture in one syringe was then mixed with an equal volume of a reaction medium without sarcoplasmic vesicles but with 11.0 mM CaCl_2 present. (The calculated free calcium concentration was, therefore, 0.64 mM using a dissociation constant of $1.9 \times 10^{-5} \text{ M}$ for Ca-EGTA complex at pH 6.0.)

with any rate, provided that during the incubation period the phosphate accepting configuration has been formed. The latter may be formed slowly either because calcium is released slowly or the protein changes its configuration slowly after calcium has been released rapidly. Distinction between the alternative mechanisms would require measurements of calcium release from the protein which however, are difficult to perform. In the second case, however, net phosphorylation of the calcium free protein should proceed not faster than phosphorylation succeeding immediately the addition of EGTA. The observation that at 20 °C the rate of phosphate incorporation into the protein after its calcium free state has been reached occurs as fast as phosphorylation following calcium removal, indicates that phosphate incorporation itself might be the rate determining step. This is true also for the slow phase of the reaction at 30 °C. The occurrence of a rapid phase of incorporation when at 30 °C the calcium free protein is phosphorylated suggests that at higher temperatures a conformation becomes apparent which interacts more rapidly with phosphate so that the formation of the phosphate accepting conformation becomes rate limiting when phosphorylation is initiated by calcium removal. The high temperature intermediate is in rapid equilibrium with medium phosphate giving rise to a two phasic phosphate exchange reaction the rapid phase of which was a half time of 10–25 msec as compared to 100–200 msec for the slow phase. An additional argument for the real existence of two different phosphorylated intermediates at temperatures above 25 °C is the two phasic decay of the phosphoprotein effected by the addition of calcium ions. This calcium induced dephosphorylation is a reaction too slow to be a step in the sequence of calcium translocation neither in the forward nor in the backward running mode of the pump. The fact that a great fraction of the phosphoprotein decays on addition of calcium 10 times slower than phosphoprotein formation proceeds during calcium removal is a most striking property of this compound. Since the calcium dependent decay of the phosphoprotein also proceeds much more slowly than its spontaneous decay, one is tempted to assume that the added calcium produces a transient stabilization of the phosphoprotein. If this long living intermediate would have become ADP-sensitive, it can possible give

rise to ATP formation provided ADP accepts the phosphoryl group sufficiently fast. This kind of reactivity of the phosphoprotein can be considered to be the basis for ATP formation by nonvesicular ATPase preparations produced by the simultaneous addition of high concentrations of calcium and ADP to the phosphorylated enzyme^{12, 13}. As to the interrelationship of the two phosphorylated intermediates in the reaction sequence being either intermediates occurring in series or in parallel, their rate of formation may be compared with the rate of ATP synthesis during calcium release. Since both rates of phosphorylation fully account for the rate of calcium release coupled ATP synthesis, no preference can be given. In any case, the obtained rate constants describing the formation of the two phosphoproteins must be considered to be much smaller than the rate constant which governs the formation of the initial protein phosphate complex (E*P). At present, the rate constant for its formation can only be guessed by comparison with similar reactions in which phosphate compounds like ATP or ATP analogues combine with sarcoplasmic membranes or other energy converting proteins^{14, 15}. Even if we choose the lowest observed rate by which these compounds combine with the enzyme, the rate constants for phosphate binding would be at least 400 times higher than the rate constants for the isomerization of the initial phosphate complex.

The enthalpy and entropy of activation for the reaction of isomerization as well as the enthalpy and entropy for phosphoprotein formation can be approximated from the temperature dependence of the rate of phosphoprotein formation and the temperature dependence of the phosphoprotein level. The values for $\Delta H = 12\,000\text{ cal}\cdot\text{mol}^{-1}$ and $\Delta S = 40\text{ e.u. mol}^{-1}$ for the equilibrium between the phosphorylated and the unphosphorylated protein which at high phosphate concentration corresponds to the ratio k_2/k_{-2} are in good agreement with those obtained by Kanazawa¹⁰ and indicate a considerable change in the conformation of the lipoprotein structure of the membranes.

The comparison of the enthalpy and entropy of activation before and after the break in the Arrhenius plot reveal a decline of the activation enthalpy from $26\,300\text{ cal}\cdot\text{mol}^{-1}$ to $17\,700\text{ cal}\cdot\text{mol}^{-1}$. At the break of the graphs at 25 °C the decline of the activation enthalpy must be compensated by a decline of the activation entropy

of 40 e.u.·mol⁻¹ in order to leave the rate constant unchanged. A break in the Arrhenius plot of the calcium dependent ATPase has been observed at the same temperature and is connected with similar changes in activation enthalpy and entropy¹⁶. Apparently, the phosphorylation of the calcium free protein with inorganic phosphate exhibits a similar kind of activity transitions as observed for the calcium dependent interaction of the transport protein with ATP.

The question as to the nature of the phosphoprotein in the native protein remains unsettled. The fact that it is bound to an amino acid residue after acid denaturation does not prove that it exists in the native state as an acyl phosphate. The covalent binding of phosphate is supported by the observed oxygene exchange between water oxygene and oxygene in the protein bound phosphate residue, that the rates of oxygene and phosphate turnover were

bound similarly by Boyer *et al.*¹⁷ strongly supports the idea that oxygene exchange is due to rapid dynamic reversal of phosphate hydrolysis and not caused by an oxygene exchange involving the penta-covalent phosphate adduct. However, these recent findings are in contrast to older results of Kanazawa and Boyer³ who observed that oxygene exchange proceeds much faster than phosphate exchange under somewhat different conditions. In any case, the applied criterion for reversible cleavage of an acylphosphate bond, namely the equality of the rate of phosphate and oxygene exchange, is a necessary but not a sufficient criterion proving the existence of a covalent bound between phosphate and a protein residue. The considerable lability of the phosphoprotein which is revealed by its rapid decay when the salt concentration in the medium is increased would imply the existence of an ionic strength sensitive acyl phosphate bond.

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