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Sarcoplasmic Reticulum Adenosinetriphosphatase Phosphorylation from Inorganic Phosphate. Theoretical and Experimental Reinvestigation†

Florent Guillain,* Philippe Champeil,‡ and Paul D. Boyer

ABSTRACT: P_i phosphorylation of sarcoplasmic reticulum (SR) vesicles in the absence of Ca was reinvestigated. Theoretical analysis shows that, for various substrate concentrations, the time dependence of phosphoenzyme formation does not allow determination of an unambiguous reaction scheme or estimation of the stoichiometry of the reaction. To overcome this difficulty, we measured medium P_i oxygen exchange, [^{32}P]-phosphoenzyme formation, and intrinsic fluorescence. We found that contrarily to the usual assumption the substrate binding step in the phosphorylation direction at pH 6.0, KCl = 0, and 23 °C is a slow process whose bimolecular rate constant is around 5×10^3 M^{-1} s^{-1} for both Mg and P_i binding. We confirm [Lacapère, J. J., Gingold, M. P., Champeil, P., & Guillain, F. (1981) *J. Biol. Chem. 256*, 2302-2306] that, in a second step, the establishment of a covalent bond between the bound P_i and the enzyme is formed with a rate constant ≥ 20 s^{-1} whereas the dephosphorylation rate constant is 2-3 s^{-1}. These results imply that under optimal conditions for phosphorylation, the enzyme is almost entirely phosphorylated at concentrations of 20 mM $MgCl_2$ and 20 mM P_i. Study of the phosphorylation reaction under various experimental conditions shows that reduction of the phosphoenzyme level upon KCl addition is mainly due to the augmentation of the hydrolysis rate constant. In addition we propose that the strong inhibition by large amounts of $MgCl_2$ is due to the formation of an $E^?$·Mg complex unfit for phosphorylation by P_i. Diminution of the phosphoenzyme level when the pH increases reflects higher enzyme sensitivity to Mg inhibition at alkaline pH. Results for inhibition by ATP of the phosphorylation reaction at pH 6.0 and KCl = 0 are also presented and discussed in the light of the data available in the literature.

One of the essential characteristics of the SR-ATPase[1] is its ability to function in a forward or a backward direction, i.e., either to accumulate calcium at the expense of ATP hydrolysis or to synthesize ATP from ADP and P_i via a phosphoryl enzyme, at the expense of an outflux of calcium.

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* Address correspondence to this author at the Département de Biologie, CEN-Saclay, 91191 Gif sur Yvette, France.
‡ Present address: Département de Biologie, CEN-Saclay, 91191 Gif sur Yvette, France.

[1] Abbreviations: SR, sarcoplasmic reticulum; ATPase, adenosinetriphosphatase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; $\Delta F/F$, relative fluorescence change.

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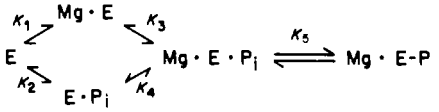
Scheme I



Phosphorylation of SR vesicles by P_i in the absence of calcium gradient was demonstrated as early as 1973, and the main properties of this partial reaction were already well documented at equilibrium (Kanazawa & Boyer, 1973; Masuda & de Meis, 1973). In 1975, Knowles and Racker showed that leaky vesicles phosphorylated by P_i were able to transfer the covalently bound phosphate from the phosphoryl enzyme to ADP for ATP synthesis (Knowles & Racker, 1975).

Subsequently there have been numerous studies of the enzyme phosphorylation from P_i at equilibrium (Masuda & de Meis, 1977; Punzengruber et al., 1978; Kolassa et al., 1979; Guimaraes-Motta & de Meis, 1980; de Meis et al., 1980, 1982; Epstein et al., 1980; Martin & Tanford, 1981; de Souza Otero & de Meis, 1982; Inesi et al., 1982; Kanazawa, 1975); the different factors affecting this reaction have also been extensively investigated, in particular, temperature (Masuda & de Meis, 1977; Epstein et al., 1980; Martin & Tanford, 1981; Kanazawa, 1975), pH (Masuda & de Meis, 1973; de Meis et al., 1980; Beil et al., 1977), KCl (Masuda & de Meis, 1973; Punzengruber et al., 1978; Shigekawa et al., 1978; Chaloub & de Meis, 1980), and ATP (Masuda & de Meis, 1973; Vieyra et al., 1979; Ariki & Boyer, 1980; McIntosh & Boyer, 1983).

From careful measurement of the amount of phosphoryl enzyme as a function of free phosphate and free magnesium, Punzengruber et al. proposed Scheme I as the most probable mechanism for the phosphorylation reaction (Punzengruber et al., 1978; Kolassa et al., 1979).

The complete solution of this scheme at equilibrium requires knowledge of the precise stoichiometry of the reaction (Punzengruber et al., 1978); however, the proportion of enzyme reactive to P_i cannot be unambiguously estimated on the basis of the values published for the amount of phosphoryl enzyme per milligram of protein, probably because of differences in SR preparations and phosphorylation conditions. Nevertheless, it has been generally assumed from kinetic studies that in the partial reaction given in Scheme II the K_3 equilibrium of the first step was fast compared to the k_5 and k_{-5} rate constants (Chaloub & de Meis, 1980; Rauch et al., 1977). With this assumption in mind, it appeared that the most straightforward way of determining the phosphorylation stoichiometry was to derive k_5 and k_{-5} from measurement of the on and off rate constants of the phosphorylation and dephosphorylation reactions. A number of such measurements have been performed but with only limited variation of the P_i and Mg concentrations. This detracts from possible reaction mechanisms proposed (Rauch et al., 1977; Lacapère et al., 1981; Inesi et al., 1982).

Scheme II

$$\text{Mg·E} + \text{P}_i \underset{}{\overset{K_3}{\rightleftharpoons}} \text{Mg·E·P}_i \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \text{Mg·E–P}$$

Because of erroneous interpretations in the literature of the kinetic properties of Scheme I, and because various stoichiometries have been postulated to fit equilibrium data, we present here additional theoretical considerations showing that determination of the time dependence of the Mg·E–P species is not sufficient to obtain a univocal kinetic scheme leading to the stoichiometry of the P_i phosphorylation of SR vesicles. To overcome this difficulty we continued here our previous kinetic study (Lacapère et al., 1981) by determining [^{32}P]-phosphoryl enzyme and by medium oxygen exchange experiments (Hackney et al., 1980). The results confirm that these exchange experiments are decisive for formulation of a nonambiguous kinetic scheme and that there is good agreement between intrinsic fluorescence and radioactive determinations. Additional results concerning the action of the principal known effectors of the dephosphorylation reaction are also presented here.

Theory

The reaction described in Scheme I is potentially confusing because it can be formulated in a variety of ways. The kinetics of formation of the Mg·E–P species would be complex if the experiments were conducted by adding both P_i and Mg at the same time. Such a complex situation can be avoided by starting with an enzyme preincubated in a medium containing P_i or Mg. Kinetically, the two cases are symmetrical, and we will only treat the case of an enzyme preincubated in an excess of P_i, as illustrated under Results. Under these conditions, Scheme I reduces to the two-step reaction of Scheme III. The overall equilibrium constant is $K_{app} = K_1(1 + K_2)$ with $K_1 = k_1/k_{-1}$ and $K_2 = k_2/k_{-2}$. For a constant Mg concentration throughout the reaction, the complete solution for the cascade of species during the time process is given by

$$X = X_\infty + X_\lambda e^{\lambda t} + X_\mu e^{\mu t}$$

where X represents one of the three species E·P_i, Mg·E·P_i, or Mg·E–P, X_∞, X_λ, and X_μ being respectively specific to each species but λ and μ common to all species.

Scheme III

$$\text{E·P}_i + \text{Mg} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Mg·E·P}_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Mg·E–P}$$

Thus, from a kinetic point of view, the only difference between the time dependence of the three species in Scheme III resides in the sign and size of the amplitudes and not in the two rate constants governing the entire system [see also Guillain et al. (1980), miniprint].

Such a conclusion means that when the SR-ATPase is preincubated in a medium containing a high P_i concentration and when the phosphorylation reaction is started by adding Mg, measurement of the time dependence of the Mg·E–P species or of any other species does not allow the observed rate constant to be identified as any one of the four rate constants of Scheme III.

This conclusion is exemplified in Figure 1, for which the set of rate constants given in the legend was chosen on the basis of the experimental data available from the literature and from the present work, i.e., at pH 6, at 23 °C, and in the absence of KCl: (i) The observed dephosphorylation rate constant is 2–5 s^{-1} (Guimaraes-Motta & de Meis, 1980; Inesi et al., 1982; Chaloub & de Meis, 1980; Vieyra et al., 1979; Rauch et al., 1977; Chaloub et al., 1979; Watanabe et al., 1981). (ii) The observed phosphorylation rate constant increases with Mg^{2+} and/or P_i concentration and reaches 10–30 s^{-1} around 20 mM (Guimaraes-Motta & de Meis, 1980; Inesi et al., 1982; Boyer et al., 1977; Chaloub & de Meis, 1980; Rauch et al., 1977; Lacapère et al., 1981; Chaloub et al., 1979). (iii) The stoichiometry of the overall reaction is between 0.5 and 1 (Punzengruber et al., 1978; Epstein et al., 1980; Martin & Tanford, 1981; McIntosh & Boyer, 1983; Lacapère et al., 1981). (iv) In the presence of high P_i concentrations, half the maximum phosphoryl enzyme is obtained for 2–5 mM $MgCl_2$ (Punzengruber et al., 1978; Epstein et al., 1980; de Meis et al., 1982; Inesi et al., 1982). (v) The appearance of the Mg·E–P species

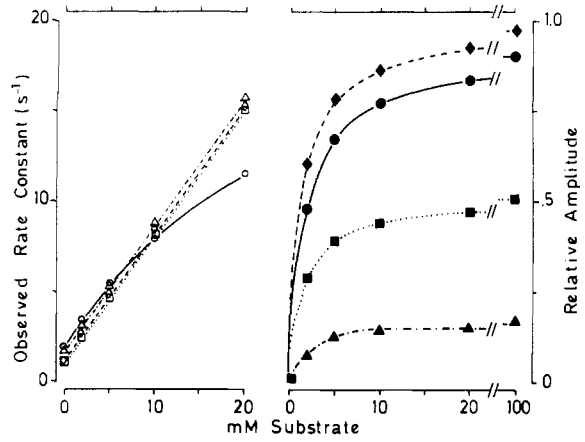


FIGURE 1: Theoretical fit for various combinations of rate constants illustrating Scheme III. The choice of numerical values for the four rate constants k_1, k_{-1}, k_2, and k_{-2} is given in the text. (○) 5000*S*, 100, 20, 2; (△) 700*S*, 2, 20, 100; (□) 700*S*, 2, 100, 100; (◇) 1000*S*, 100, 100, 2. Only (△) can be rejected because of unduly low stoichiometry.

is almost monoexponential (Lacapère et al., 1981).

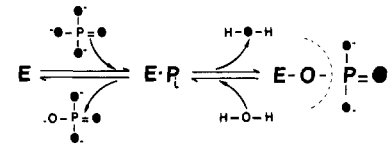
In Figure 1 three of the four possible combinations deduced from the chosen set of rate constants give results which cannot be distinguished with a reasonable margin of experimental error. For example, a slow rate constant in the reversal direction of the reaction producing a slow observed off rate can be chosen either for the dissociation step (square) or for the hydrolysis step (diamond) without any apparent difference in the observed rate constant but resulting in half (filled square) or nearly full (filled diamond) phosphorylation. Figure 1 of course refers to only one set of numerical values, but the choice of the four rate constants is not the only one giving very similar k_{obsd} values for the phosphoryl enzyme formation from P_i.

Important information can be derived from the plot of the k_{obsd} values when it displays a marked curvature or tends to a limiting k_{obsd} value for high substrate concentrations: since the rate of the association step is a linear function of the substrate concentration, this first step is very fast for high substrate concentrations, and the second step then becomes the rate-limiting step. Therefore, the limit of the k_{obsd} value at infinite substrate concentration—or in practice, the curvature of the k_{obsd} plot for high substrate concentrations—is characteristic of the second step whose theoretically limiting observed rate constant is $k_2 + k_{-2}$.

This brief theoretical analysis shows that in addition to the overall equilibrium constant, measurement of the rate of Mg·E–P formation for several substrate concentrations is not sufficient to identify the four rate constants in Scheme III and is therefore also insufficient to determine the stoichiometry of the reaction. To overcome this difficulty, more information is required. This can be provided by medium P_i oxygen exchange measurements.

Oxygen exchange results from reversal of the step in which an oxygen atom of water is incorporated into P_i when the phosphoryl enzyme is hydrolyzed. Scheme IV illustrates such hydrolysis for an enzyme phosphorylated by $P^{18}O_4$ (i.e., by phosphate containing 100% ^{18}O) in a medium containing unlabeled water (i.e., $H_2{}^{16}O$). Precise location of an elementary reaction in the entire process allows identification of at least one rate constant. After hydrolysis, the Mg·E·P species may either reform into Mg·E–P (k_2) or release P_i into the medium (k_{-1}) and each P_i molecule might undergo several oxygen exchanges, giving a pool of different phosphate species containing various amounts of ^{18}O. The distribution of these different ^{18}O–P_i species as a function of time is characteristic

Scheme IV



of the reaction, and the partition coefficient $P_c = k_2/(k_{-1} + k_2)$ gives the probability that Mg·E·P_i will enter the exchange step (Hackney et al., 1980). Depending on the relative magnitude of the k_{-1} and k_2 rate constants, the apparent hydrolysis rate constant (k_{off}), measured by the disappearance of the phosphoryl enzyme, may be significantly slower than the actual hydrolysis rate (k_{-2} governed). The ratio of the measured k_{off} rate constant to k_{-2} represents the probability of a dissociation rather than an oxygen exchange, i.e., $1 - P_c$, and therefore

$$k_{off} = k_{-2}(1 - P_c)$$

Under the conditions of the present studies, as will be seen below, $P_c \simeq 0$; whenever an Mg·E–P molecule is hydrolyzed, the resulting Mg·E·P_i dissociates; i.e., each Mg·E–P hydrolysis leads to incorporation of one water oxygen into medium P_i, and the rate of total exchange of P_i oxygens (v_{ex}) allows calculation of k_{-2} by the simple relation (Hackney et al., 1980)

$$V_{ex} = k_{-2}(\text{Mg·E–P}) = k_{av}[4(P_i)]/(\text{protein concentration})$$

Therefore, oxygen exchange and Mg·E–P determinations allow calculation of the true hydrolysis rate constant, k_{-2}, and of the ratio $k_2/(k_{-1} + k_2)$. If the values found for k_{obsd} as a function of Mg, K_{app}, k_{-2}, and P_c are pooled, we can calculate and identify all the rate constants of Scheme II, as shown under Results. Note, however, that this does not mean that Scheme III is the only kinetic model fitting the experimental data.

Materials and Methods

The experimental procedures used for SR vesicle preparation and kinetic and equilibrium fluorescence measurements have already been described in Champeil et al. (1978) and Guillain et al. (1982). Multimixing and steady-state [^{32}P]phosphoryl enzyme measurements are given with the free ion concentration calculations in Lacapère et al. (1981). The present experiments were carried out at 23 ± 1 °C to obtain maximal phosphorylation (Kanazawa, 1975; Masuda & de Meis, 1977); media are described in the legends and P_i was added as Tris salt. Medium oxygen exchange experiments were conducted as described in McIntosh & Boyer (1983).

Results

Fluorescence and ^{32}P Measurements. In order to follow by fluorescence the kinetics of the phosphorylation reaction, it was important to show that intrinsic fluorescence was a good index of that reaction. In a previous work (Lacapère et al., 1981) we showed that there was a very good parallel between fluorescence and ^{32}P determinations at equilibrium. In the present work, Figure 2 shows on and off rate constant determinations made by two different methods under the same experimental conditions, i.e., pH 6 and in the absence of KCl. The circles represent the amounts of ^{32}P-labeled phosphoryl enzyme determined by the rapid quenching method, and the continuous trace shows transient SR-ATPase intrinsic fluorescence. As in the case of the equilibrium conditions, there was a good overlap of the two methods.

Figure 3 shows a plot of the observed rate constant (k_{obsd}) as a function of the free Mg^{2+} concentration. This Mg^{2+}

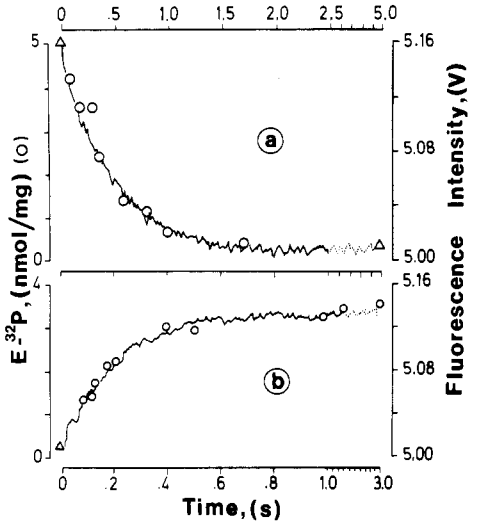


FIGURE 2: Simultaneous recording of P_i phosphorylation by ^{32}P incorporation and intrinsic fluorescence. General medium: 150 mM MES–Tris and 2 mM EGTA, pH 6.0. Concentrations are expressed as final concentration after mixing and as free concentrations for P_i and Mg. (a) *Off measurements*: initial concentrations 10 mM $MgCl_2$ and 10 mM P_i. Radioactivity measurements were carried out with 0.5 mg of protein by ^{32}P incorporation into the initially nonradioactive Mg·E–P species. Fluorescence was measured at 0.05 mg/mL protein and by Mg chelation, by mixing 20 mM EDTA. *On measurements*: at time zero, same conditions as for off measurements, except that $MgCl_2$ = 0 and $[^{32}P]P_i$ was used for multimixing. Both phosphorylations were induced by mixing 3.3 mM $MgCl_2$.

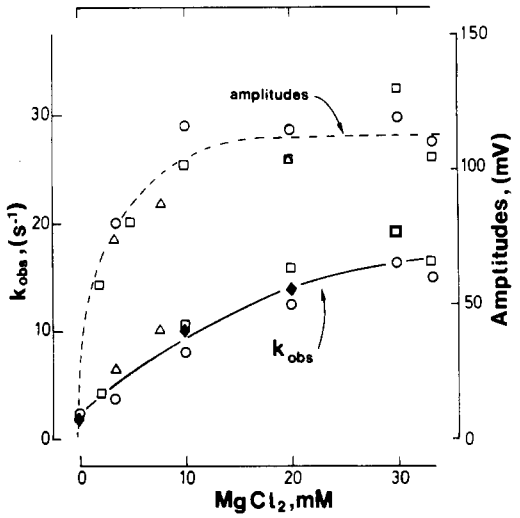


FIGURE 3: Magnesium concentration dependence of the kinetic parameters of P_i phosphorylation. General conditions as for on measurements in Figure 2. Closed symbols are ^{32}P determinations by the multimixing technique. Open symbols are stopped-flow determinations for various SR preparations with a 5-V signal at time zero.

dependence was chosen because ^{32}P measurements are difficult to carry out for P_i concentrations higher than 10 mM, whereas at a given low P_i concentration, the k_{obsd} for increasing Mg concentrations can be evaluated up to the limit of the multimixing apparatus, i.e., $k_{obsd} \simeq 10\ s^{-1}$. To explore with adequate precision the reactions when k_{obsd} exceeded 10 s^{-1}, it was necessary to use the stopped-flow technique, whose upper limit is around 100 s^{-1}. The agreement in Figure 3 between ^{32}P incorporation and fluorescence measurements confirms our previous results; i.e., starting from an off rate constant of 2 s^{-1}, k_{obsd} increases with the Mg concentration. This is also in agreement with results recently presented by Inesi et al. (1984).

However, as discussed under Theory and contrary to what has been assumed so far, the rate constants in Figure 3 cannot

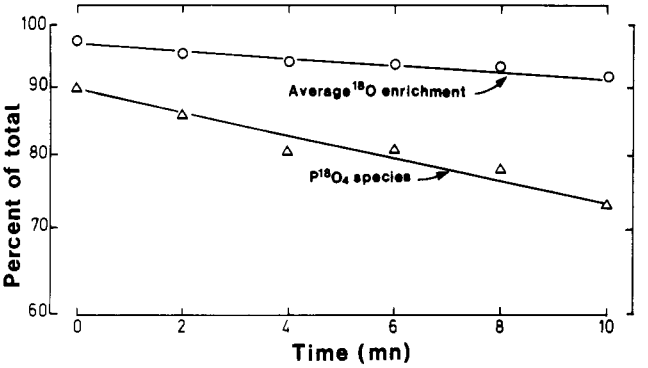


FIGURE 4: Time course of the $P_i \rightleftharpoons H_2O$ oxygen exchange catalyzed by SR vesicles at pH 6.0 in the absence of KCl. (Δ) Decrease of the $P^{18}O_4$ species. (O) Average loss of ^{18}O taking into account all P_i isotopes (see text). Experimental conditions as in Figure 6. Oxygen exchange measurements as described in McIntosh & Boyer (1983).

Table I: pH and KCl Dependence of Various Parameters on the Phosphorylation Reaction at 23 °C

pH	KCl (mM)	Mg·E–P[a] (nmol/mg)	P_c	k_{-2}[b] (s^{-1})	off[c] (s^{-1})	on[d] (s^{-1})
6.0	0	5.5	0.15	3	2	20
	100	3.0	0.15	12	15	40
6.5	0	4.6	0.12	3.5	4	50
	100	2.5	0.08	27	40	70
7.0	0	2.5	0.10	25		
	100	1.4	0.07	70		

[a] Phosphoryl enzyme levels were evaluated by phosphorylation of 1 mg of protein in the presence of 20 mM $MgCl_2$ and 20 mM $[^{32}P]P_i$ (free concentrations). [b] Rate constants calculated by oxygen exchange measurements. [c] Intrinsic fluorescence measurements by Mg chelation (see text). [d] Intrinsic fluorescence measurement at 20 mM P_2 and 20 mM $MgCl_2$ as final free concentrations.

be identified with any of the four constants of Scheme III. The only straightforward results deducible from the present kinetic experiments are an apparent off rate constant of 2 s^{-1}, an augmentation of the on rate constant with the substrate concentration (the k_{obsd} plot being slightly curved for high Mg concentrations) and 500 M^{-1} for the overall association constant.

Medium Oxygen Exchange Measurements. Figure 4 shows an example of the time dependence of the loss of $P^{18}O_4$ (triangles), as well as of the average loss in ^{18}O, taking into account all ^{18}O–P_i species[2] (circles). A much steeper slope for the variation in $P^{18}O_4$ indicates a small P_c.[3] This means that each hydrolysis (Mg·E–P → Mg·E·P_i) is followed by dissociation of the newly formed P_i molecule (Mg·E·P_i → Mg·E + P_i), and this low P_c allows calculation of the true hydrolysis rate constant by the relation

$$k_{-2} = k_{av}[4(P_i)]/[(Mg{\cdot}E{-}P)(\text{protein concentration})]$$

The results obtained under different experimental conditions have been collected in Table I. The data for pH 6 in the absence of KCl give k_{-2} = 3 s^{-1}, a value similar to the one deduced from the multimixing and fluorescence results (Figure 2a). Therefore, the off rate constant measured by fluorescence or ^{32}P incorporation in Figure 3 can now be identified as the hydrolysis rate constant slightly modified by the dissociation step [$k_{off} = k_{-2}(1 - P_c)$].

[2] When the amounts of the different P_i isotopes are expressed in percent, the average ^{18}O content is $^1/_4(4P^{18}O_4 + 3P^{18}O_3 + 2P^{18}O_2 + 1P^{18}O_1)$.

[3] If k_4 and k_{av} are the respective rate constants for the loss of $P^{18}O_4$ and for the loss of average ^{18}O (Hackney et al., 1980), then $P_c = {}^1/_3(4 - k_4/k_{av})$.

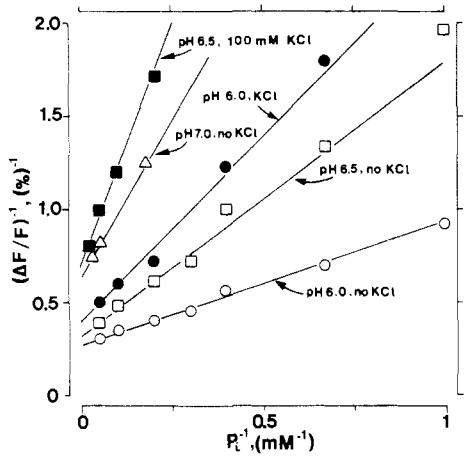


FIGURE 5: Reciprocal plot of the phosphorylation-induced fluorescence changes. Experimental conditions as in Figure 6 except for variable amounts of P_i and 150 mM MOPS–Tris as buffer at pH 7.0. (●, ○) pH 6.0; (■, □) pH 6.5; (△) pH 7.0; (○, □, △) KCl = 0; (●, ■) KCl = 100 mM.

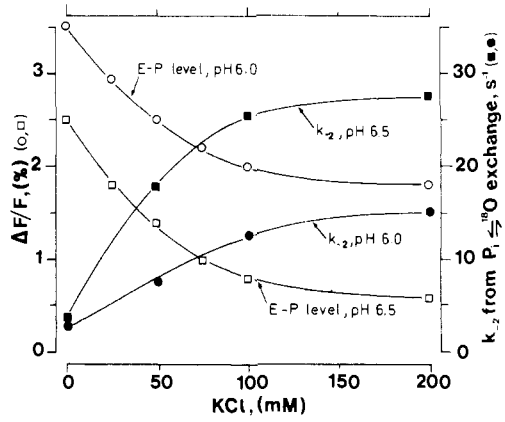


FIGURE 6: KCl dependence of the phosphorylation-induced fluorescence change and of the k_{-2} rate constant deduced from oxygen exchange measurement. General medium: 150 mM MES–Tris, 2 mM EGTA, 20 mM $MgCl_2$, and 20 mM P_i or $P^{18}O_4$ (total concentrations). (●, ○) pH 6.0; (■, □) pH 6.5.

If Figure 3 is now considered in the light of the unambiguous values k_{-2} = 2–3 s^{-1}, P_c = 0.15, and K_{app} = 500 M^{-1}, the curvature of the k_{obsd} plot leads to $k_2 \geq 20$ s^{-1}, a value giving a reasonable fit according to the differential equation system derived from Scheme III. Finally

$$k_{-1} = k_2(1 - P_c)/P_c \simeq 100 \text{ s}^{-1}$$

$$k_1 = (K_{app}k_{-1}k_{-2})/(k_2 + k_{-2}) \simeq 5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$$

Bearing in mind that the P_c value and the k_{obsd} value for high substrate concentrations are independent of the order of substrate addition, and if, as shown below, we anticipate that P_i and Mg have similar overall equilibrium constants [compare Figure 3 (amplitudes) to Figure 5 (open circles)], the above calculations for Mg binding to vesicles preincubated in the presence of P_i also hold for calculation of the rate constants for P_i binding to vesicles preincubated in the presence of Mg.

Therefore, in the on direction, the binding process may, for a low millimolar substrate, be slower than the establishment of the covalent bond. This is a surprising result, and the former hypothesis of a fast binding step resulted from a coincidental combination of circumstances allowing good fits of kinetic and equilibrium data, but giving a wrong image of the phosphorylation reaction. It is worth mentioning that, when the K_2 equilibrium is taken into account, the phosphorylation stoichiometry is

$$(\text{Mg·E–P})_{max} \geq 0.9E_{total}$$

which corresponds to almost complete phosphorylation under these conditions (pH 6.0, KCl = 0; see Table I).

KCl Dependence of Phosphorylation at pH 6. The numerous studies devoted to the effect of potassium on the phosphorylation reaction show that KCl strongly reduces the phosphoryl enzyme level. In this respect, the first effect to stress from the present results is that pH and KCl do not drastically change P_i affinity. In Figure 5, the P_i affinities lie between 2 and 10 mM for different pH and KCl combinations. The parallel between Mg·E–P reduction and k_{-2} augmentation in Figure 6 clearly shows that the main effect of KCl is to diminish the phosphoryl enzyme level by accelerating the k_{-2} rate constant. Furthermore, when, in a stopped-flow experiment, appropriate spectral and experimental conditions were chosen to avoid fluorescence perturbations and/or osmotic shock (Guillain et al., 1982; Champeil et al., 1983), rapid mixing of large amounts of KCl to an Mg·E–P species induced a fast drop in intrinsic fluorescence. This drop occurred with a rate constant similar to the one measured in the presence of KCl when dephosphorylation was induced by chelation of Mg^{2+} ions by EDTA (data not shown). This observation suggests that inhibition by KCl is a fast process, even faster than the off direction of the reaction. However, in terms of stoichiometry, ^{32}P determinations of the Mg·E–P levels are slightly at variance with what would be expected from fluorescence measurement of on and off rate constants [$s = \text{Mg·E–P}_{max}/E_{total} = k_2/(k_2 + k_{-2})$], and the simple explanation given above for the Mg·E–P reduction upon KCl addition is insufficient, particularly when pH was >6.0. For example, in Table I, at pH 6.5 and KCl = 100 mM, the phosphoryl enzyme level gives s = 0.4, a value which predicts k_2 = 18 s^{-1}, whereas the on fluorescence measurement gives $k_2 \geq 70$ s^{-1}. This discrepancy is far larger than the possible experimental error and indicates a more complex situation when pH was >6.0. We now report several experiments describing this new situation.

Complex Situation When pH Was >6. Table I shows that, for all pHs studied, the presence of KCl increased the k_{-2} rate constant, and the k_{-2} value determined at pH 6.5 and 100 mM KCl is in good agreement with the k_{-2} = 51 s^{-1} determined by McIntosh & Boyer (1983) under very similar experimental conditions at 25 °C, despite the lower phosphoryl enzyme level they reported.

However, the first manifestation of a complex situation at pHs higher than pH 6 was that, when the enzyme was phosphorylated at pH 7, the fluorescence rose but that, when 100 mM KCl was added, fluorescence returned to its original level (Figure 7) even though under similar experimental conditions, ^{32}P incorporation revealed the presence of a definite amount of phosphoryl enzyme (Table I). Another indication of a complex situation when pH was >6 was the stronger inhibition of the phosphoryl enzyme level by large amounts of $MgCl_2$. Such inhibition of phosphorylation from P_i has already been described by Loomis et al. (1982), and in Figure 8, the fluorescence change induced by phosphorylation of the vesicles when 20 mM free P_i was kept constant was plotted over a wide range of $MgCl_2$ concentrations. Here again, the spectral conditions for the analysis were chosen so that the Mg^{2+}-induced fluorescence rise per se did not perturb the measurement. Under those circumstances, the Mg·E–P level was clearly maximum around 10 mM and sharply declined after further addition of $MgCl_2$, this drop being much more sensitive to Mg at pH 6.5 than at pH 6.0. Furthermore, as

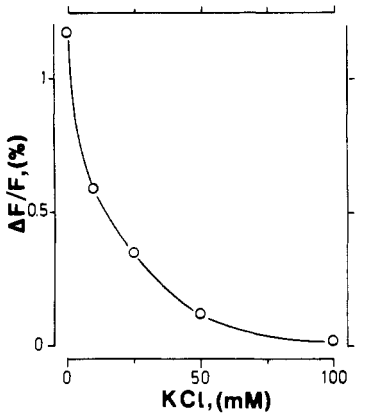


FIGURE 7: Decrease at pH 7.0 of the phosphorylation-induced fluorescence change upon KCl addition. General medium: 150 mM MOPS–Tris, 2 mM EGTA, 50 μg/mL protein, 10 mM $MgCl_2$ and 20 mM P_i (free concentrations). Spectral conditions: λ_{ex} = 290 nm; λ_{em} = 345 nm. To exclude Mg^{2+}-induced fluorescence artifacts, the experiment was checked with various λ_{ex} and λ_{em} combinations (Guillain et al., 1982). At 100 mM KCl, no fluorescence change was detected upon P_i addition.

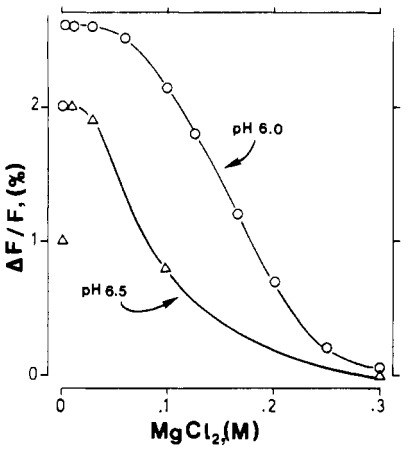


FIGURE 8: Inhibition of the P_i phosphorylation by high Mg concentrations. (○) pH 6.0; (△) pH 6.5. Buffer conditions as in Figure 6, and 20 mM P_i constant free concentration. Spectral conditions as in Figure 7.

we saw above for KCl, dephosphorylation can be induced by rapid mixing of large amounts of $MgCl_2$ (data not shown), and here again, the observed rate constant was fast. This points to the dual effect of Mg^{2+} ions on the simple phosphorylation reaction, in which Mg is an absolute requirement for phosphorylation but accelerates the reverse reaction at inhibiting concentrations. Note, however, that the two inhibitions shown in Figure 8 occurred in less than one decade. As will be discussed below, this suggests a more complex inhibition mechanism than simply the acceleration of the dephosphorylation rate constant.

Effect of ATP on the Off Rate Constant. Finally the most probable effector of the partial phosphorylation reaction during the entire ATPase cycle is the ATP itself. This has been studied by McIntosh & Boyer (1983), who found a strong modulation of the off rate constant. Here, we present data obtained under restricted experimental conditions at pH 6.0 and in the absence of KCl.

Irrespective of the presence or absence of Mg in the medium, ATP bound to SR vesicles even when the ATPase was in its P_i-phosphorylable conformation, i.e., in a Ca-deprived state. In Figure 9, where binding was measured by the filtration technique (Dupont, 1980), the maximal amount of bound [^{14}C]ATP, extrapolated to infinite concentration, was around

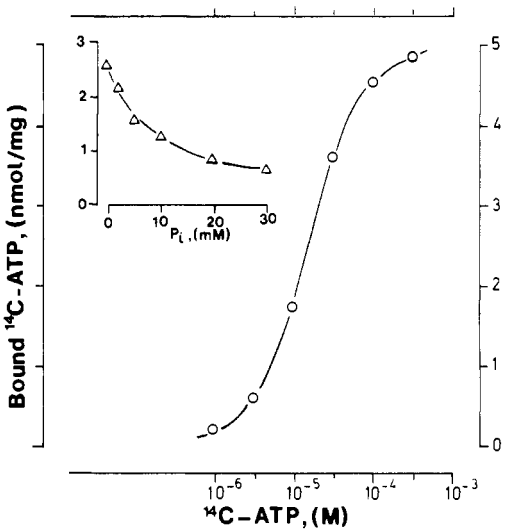


FIGURE 9: ATP binding (○) and P_i inhibition (△) of ATP binding at pH 6.0, in the absence of KCl. Each assay was carried out by Millipore filtration and scintillation counting of 0.5 mg of protein (Dupont, 1980) under the buffer conditions described in Figure 6 and with 20 mM free Mg^{2+} and various amounts of [^{14}C]ATP. (Inset) 20 mM free Mg^{2+}, 20 μM [^{14}C]ATP, and various amounts of P_i.

6 nmol/mg and the corresponding association constant, 6×10^4 M^{-1} (data in the absence of Mg not shown). The inset in Figure 9 further shows that phosphorylation of the enzyme inhibited ATP binding. When compared to the overall equilibrium constant K_{P_i} = 400 M^{-1} found for the P_i affinity at pH 6.0 and KCl = 0 (Figure 5, open circles), the inhibition constant K_I = 200 M^{-1}, which can be deduced from Figure 9 inset, suggests a competitive inhibition between P_i phosphorylation and ATP binding. When we used the stopped-flow technique to study the dephosphorylation induced by Mg^{2+} chelation in the presence of various amounts of ATP (0–100 μM), we did not find any measurable influence of ATP (data not shown). To maintain a constant Mg concentration during the dephosphorylation process, we also induced the off reaction by rapid mixing of 100 μM ATP (final concentration) with phosphoryl enzyme, and here again, the observed rate constant was very similar to the one measured in Figure 2a.

Discussion

Theory section shows that equilibrium and kinetic studies of Mg·E–P formation alone do not allow determination of the stoichiometry of the phosphorylation reaction. However, the extent of phosphorylation is an important parameter giving fundamental indications about the Ca^{2+} ion pumping mechanism. For instance, some explanations proposed to account for experimental results include half-reactivity (Ikemoto et al., 1981; Dupont, 1982) as well as partial denaturation of the enzyme (Takisawa & Makinose, 1981; Murphy et al., 1982).

To supplement these insufficient equilibrium and kinetic results, we combined fluorescence or ^{32}P investigations with oxygen exchange experiments. Oxygen exchange enables characterization of the hydrolysis step, as well as calculation of the binding rate constants. Collection of the results presented in this report makes it possible to summarize phosphorylation at pH 6.0 in the absence of KCl as follows: during the binding process, the on rate constant ($k_1 = 5 \times 10^3$ M^{-1} s^{-1}) does not allow fast substrate binding in the millimolar range, whereas the dissocation rate constant (k_{-1} = 100 s^{-1}) can be considered fast compared to the Mg·E–P hydrolysis step. Furthermore, the establishment of a covalent bond between phosphate and SR-ATPase is a favorable process ($k_2 \geq 20$ s^{-1}, k_{-2} = 2 s^{-1}). This implies that at pH 6.0 and KCl

= 0, the enzyme is almost entirely phosphorylable by P_i. In fact, we measured high Mg·E–P levels (Table I) which agreed both with the number of ATP binding sites measured in Figure 9 and with the number of polypeptide chains, taking 115 000 as the molecular weight of the SR-ATPase and 70–80% as the ATPase content of our preparations (Champeil et al., 1978).

The present data also confirm some important points of previous fluorescence results, particularly the lack of synergism for Mg and P_i binding. In Scheme I, K_3 and K_4 can only be determined if K_5 is known, and the different previous calculations showing synergistic binding of Mg and P_i were carried out assuming that $K_5 \simeq 1$. It is worth mentioning that kinetic evaluation of the stoichiometry has been also attempted, but only at 2 mM P_i, a concentration which was obviously too low for a limit evaluation at infinite P_i. A more detailed discussion about the validity of such experimental evaluations can be found in Lacapère et al. (1981).

The second essential result of the present work is that it clarifies the complex dependence of the phosphorylation reaction on the pH and the KCl and Mg concentrations. In this respect a surprising result is the poor change in affinity of the enzyme for P_i induced by KCl and/or increasing pHs (Figure 5), which contrasted sharply with the drastic additive effects of these two factors on the phosphoryl enzyme level (Figure 6). Roughly speaking, KCl accelerated hydrolysis at all the pHs studied [see k_{-2} augmentation in Table I and Chaloub & de Meis (1980)], and alkaline pHs made the enzyme very sensitive to Mg^{2+} inhibition. Such inhibition of P_i phosphorylation has been already described by Loomis et al. (1982), and a similar Mg^{2+} inhibition has been reported for Ca binding to the SR-ATPase (Guillain et al., 1982; Champeil et al., 1983). At the time, we explained our results by suggesting that Mg binding drives the enzyme toward an E'·Mg conformation, which is unfit for P_i phosphorylation, and mimics the $E{\cdot}Ca_2$ conformation (the E'·Mg species is different from the Mg·E one in Schemes I–III). The amount of enzyme available for phosphorylation is therefore $E_{total} - E'{\cdot}Mg$. Assuming a constant free P_i concentration, this decline in phosphorylation can be summarized by Scheme V.

Scheme V

$$E'{\cdot}Mg \overset{K_{Mg}}{\rightleftharpoons} E + Mg \overset{K_{app}}{\rightleftharpoons} Mg{\cdot}E{-}P$$

We previously demonstrated that K_{Mg} rises with the pH (Guillain et al., 1982), and this result does in fact predict a lower apparent stoichiometry when the pH increases, even if the phosphorylation remains favorable (i.e., $k_2 > k_{-2}$ in Scheme III). Nevertheless, although there is a good correlation between the K_{Mg} augmentation and the stoichiometry diminution when the pH increases, Scheme V does not predict the total inhibition shown in Figure 9, particularly at pH 6.0 where K_{Mg} = 36 M^{-1} (Guillain et al., 1982) and K_{app} = 500 M^{-1} (Figure 3). This contradiction is consistent with the complex inhibition mechanism proposed by Loomis et al. (1982) in which several Mg^{2+} ions drive the enzyme away from the conformation phosphorylable by P_i.

Inesi et al. (1984) recently proposed an alternative explanation for inhibition of the phosphoryl enzyme formation at alkaline pH. They pointed out that any change of pH of the medium leads to a change in both substrate and enzyme ionization. Assuming a set of equilibrium constants for the various possible binding combinations between three enzyme states and two forms of substrate, they obtained a reasonable fit for the Mg·E–P level as a function of the substrate concentration. Because the concentration of the Mg·P_i species also increases with pH, a very straightforward extension of this suggestion would be to consider the Mg·P_i complex as an inhibitor of the phosphorylation reaction.

However, we do not consider that these explanations are the most probable for two reasons: (i) as mentioned above, alkaline pH favors interaction of the enzyme with Mg^{2+} ions (Guillain et al., 1982), and this phenomenon is reasonably interpreted as a depletion of the P_i phosphorylable form of the enzyme (Martin & Tanford, 1981; Loomis et al., 1982). Further, we have kinetic evidence for the validity of this hypothesis (P. Champeil et al., unpublished results). (ii) In the presence of dimethyl sulfoxide, the Mg·P_i complex is a serious candidate for being the true substrate and not an inhibitor of the phosphorylation reaction (P. Champeil et al., unpublished results). In that respect, this might explain why phosphate and magnesium, which are substrates with opposite charges, have very similar affinities. Furthermore, when the pH increases, the augmentation of the Mg·P_i concentration could be antagonized by stronger inhibition of the Mg^{2+} ions, and this could be an explanation for the poor apparent sensitivity of P_i and Mg^{2+} binding to the pH.

Finally, the third aspect we would like to discuss is the modulation of the reaction by ATP. Here we showed that, at pH 6.0 and KCl = 0, ATP did not affect the observed off rate constant; moreover, other data obtained by ^{32}P incorporation show the same ATP insensitivity at pH 7.0 in the absence of KCl (unpublished results). The present results further show that, at least at pH 6.0 and KCl = 0, P_i phosphorylation and ATP binding are mutually exclusive. This contrasts with the results obtained by McIntosh & Boyer (1983) under conditions more relevant to the entire ATPase cycle (pH 6.5 and 50 mM KCl), which support a strong modulation of dephosphorylation by ATP. This discrepancy either resides in the different experimental conditions or might suggest that, when the enzyme is in its phosphorylated form in the absence of KCl, no ATP binding site is available for a modulating ATP molecule. On the contrary, the presence of KCl pulls the enzyme into a different phosphorylated conformation, thus unmasking a modulator ATP binding site. This might explain the lack of change in fluorescence upon phosphorylation in the presence of 100 mM KCl (Figure 7).

Acknowledgments

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Registry No. ATPase, 9000-83-3; phosphate, 14265-44-2; magnesium, 7439-95-4.

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Electron-Transfer Reactions of Photoreduced Flavin Analogues with *c*-Type Cytochromes: Quantitation of Steric and Electrostatic Factors†

T. E. Meyer, J. A. Watkins, C. T. Przysiecki, G. Tollin, and M. A. Cusanovich*

ABSTRACT: We have found correlations between rate constants and the difference in redox potential of the reactants for electron-transfer reactions between oxidized cytochromes and either photoproduced riboflavin or flavin mononucleotide (FMN) semiquinones (the latter rate constants extrapolated to infinite ionic strength). The riboflavin–cytochrome rate constants are about 70% of those for reduction by lumiflavin, probably because of steric interference by the ribityl side chain. Reduction of cytochromes by FMN semiquinone was ionic strength dependent in all cases, due to electrostatic interactions. Extrapolation of rate constants to infinite ionic strength shows that the phosphate exerts a significant steric effect as well (rate constants average about 27% of those for lumiflavin, although part of this decrease is due to a difference in the semiquinone p*K* value). Differences in the magnitude of the FMN steric effect correlate well with surface topology differences for those cytochomes whose three-dimensional structures are known. Mitochondrial cytochromes *c* and the cytochromes c_2 all showed attractive (plus–minus) interaction with FMN in spite of the fact that some of these proteins have large net negative charges. Four small *c*-type cytochromes (including *Pseudomonas* cytochrome *c*-551) show a weak repulsive interaction with FMN semiquinone. We conclude that flavosemiquinones interact at a site on the cytochromes that is near the exposed heme edge. There is a large positive electrostatic field at this site in mitochondrial cytochrome *c* and the cytochromes c_2, but this region is primarily hydrophobic in *Pseudomonas* cytochrome *c*-551 and in the other small bacterial cytochromes. In the *Pseudomonas* reaction, FMN interacts weakly with a negative electrostatic field, which must be somewhat removed from the site of electron transfer. The relative contributions of redox potential, steric effects, and electrostatic interactions for the flavosemiquinone–cytochrome reactions described herein are roughly equivalent and correspond to rate constant changes of 2–5-fold.

We have been using flavin semiquinones generated by laser flash photolysis as reductants to study electron-transfer mechanisms in cytochromes and other redox proteins (Ahmad et al., 1981; Meyer et al., 1983). Theoretically, one would expect that redox potentials, steric factors, and electrostatics should all contribute to rate constants for the reaction, but until recently, the quantitative contributions of these factors have not been demonstrated. By using a large set of homologous

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