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PHOSPHORYLASE KINASE: DEVELOPMENT OF A CONTINUOUS FLUOROMETRIC ASSAY FOR THE DETERMINATION OF CATALYTIC ACTIVITY

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SUMMARY: The preferential binding of 1-anilinonaphthalene-8-sulfonate by rabbit muscle phosphorylase *a* is the basis of a continuous fluorometric assay for phosphorylase kinase. The maximum rate of change in fluorescence ($d\Delta F/dt$) is dependent on both the concentration of phosphorylase kinase and on conditions, such as pH and calcium ion concentration, which affect the enzyme. Parallel measurements of the increases in fluorescence and of ^{32}P incorporation demonstrate the existence of a distinct intermediate in the conversion of phosphorylase *b* to *a*. We have used the assay to monitor the increase in calcium-independent activity which accompanies the limited chymotryptic digestion of phosphorylase kinase.

Rabbit skeletal muscle phosphorylase kinase, which catalyzes the conversion of phosphorylase *b* to *a*, is subject to dual regulation by calcium and cAMP (cf. reviews in 1,2,3,4). It is a high molecular weight complex (1.3×10^6) containing four sets of four different subunits: $\alpha_4\beta_4\gamma_4\delta_4$. The γ subunit (45-kD) contains the catalytic site; the δ subunit (calmodulin, 17-kD) binds calcium; the β subunit (125-kDa) contains the phosphorylation site that is involved in the activation of the enzyme by cAMP-dependent protein kinase; and the α subunit (138-kDa) is of largely unknown function. Routine assays of phosphorylase kinase monitor the concentration of phosphorylase *a* in a two-step incubation procedure, involving final measurement of the inorganic phosphate released during the *in vitro* synthesis of glycogen from glucose-1-phosphate (5,6). Drawbacks to this method include its discontinuous nature and the errors inherent in the dilutions and transfers that are made.

Earlier work from our laboratory provides the basis for a continuous fluorometric assay of phosphorylase kinase. Rabbit muscle phosphorylase undergoes a rapid, reversible association with the fluorescent dye, 1-anilinonaphthalene-8-sulfonate (ANS) (7). At low concentrations of ANS in solutions

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Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; G-6-P, glucose-6-phosphate.

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containing 20 mM Tris, the amount of dye bound by the phosphorylated form of the enzyme (a) is two to three times greater than that bound by phosphorylase b. In addition, the binding is affected by substrates and allosteric effectors of phosphorylase: e.g., AMP, glucose-6-phosphate, and glucose-1-phosphate (8). X-ray crystallographic studies of phosphorylase b showed that ANS binds primarily to the activator site, in competition with AMP (9).

The results in this article establish a sensitive catalytic assay of phosphorylase kinase based on continuous measurement of the increases in ANS fluorescence that accompany the phosphorylase b to a conversion. The applicability of the assay is demonstrated in experiments with both native rabbit muscle phosphorylase kinase and its 33-kD catalytically active chymotryptic fragment.

MATERIALS AND METHODS

Rabbit muscle phosphorylase b (10) and a (5), phosphorylase kinase (11), and the catalytic subunit of beef heart cAMP-dependent protein kinase (12) were obtained following standard techniques. The catalytically active chymotryptic fragment (1) was purified by a non-HPLC procedure (in preparation). 1-Anilinonaphthalene-8-sulfonate was recrystallized as the magnesium salt (13). ATP, dithiothreitol, glucose-6-phosphate, disodium glycerophosphate, EGTA, and Tris were purchased from Sigma Chemical Company. [γ-^{32}P]ATP (3000 C_i/mmol) was obtained from New England Nuclear. All other chemicals were either reagent or highest available grade. Solutions were prepared with de-ionized water that had been further purified with a Milli-Q reagent water system. Before use in the assay, phosphorylase samples were charcoal-treated to remove traces of AMP.

The buffer and substrate concentrations employed in our assay were adopted, with modifications, from the conditions used in the original two-stage assay (5,6). Solutions contained 50 mM glycerophosphate and 50 mM Tris, adjusted to either pH 6.8 or pH 8.3 with HCl. Since this buffer is subject to deterioration, it was freshly prepared each week and stored at -70°. Solutions also contained 1.0 mM ATP, 6.0 mM magnesium acetate, 1.0 mM dithiothreitol, 2.6 mg/mL phosphorylase b, and either 1.0 mM EGTA or 0.1 mM $CaCl_2$. The concentration of ANS employed (20 µM) was based on the results of previous binding studies, which showed that major differences between phosphorylase b and a would be evident (8).

The fluoresence assays were monitored in a Hitachi Perkin Elmer MPF-2A fluorometer connected to a circulating constant temperature bath. Quartz fluorescence cuvettes (1-cm path) were filled with 1.6 mL of solution. The excitation wavelength was fixed at 360 nm and the emission wavelength at 460-470 nm. The latter range of wavelengths minimizes the background fluorescence due to phosphorylase alone. The relative change in fluorescence (ΔF) is calculated from

$$(F_{obs}-F_{phos\ b})/F_{phos\ a}-F_{phos\ b}).$$

$F_{phos\ b}$ is the fluorescence at zero time, $F_{phos\ a}$ is the fluorescence measured at equilibrium, and F_{obs} is the fluorescence observed at intermediate times. The values of $F_{phos\ a}$ were corroborated in separate measurements with purified phosphorylase a.

Parallel assays of ^{32}P incorporation were performed on 300 µL samples. The ATP was adjusted to a specific radioactivity of 0.02 C_i/mmol. After varying incubation times, 20 µL aliquots of the reaction mixtures were applied to P81 phosphocellulose paper (Whatman) and washed successively with 75 mM H_3PO_4, 95% ethanol, and ether. The radioactivities of the dried samples were determined

using scintillation fluid. Nearly total recovery (>95%) of phosphorylase can be obtained with P81 phosphocellulose. (Yields of only 30-40% were found with GFB glass fiber filters.)

RESULTS

Figs. 1 and 2 illustrate the time-dependent fluorescence intensity changes occurring when either native phosphorylase kinase or the catalytically active fragment is added to the assay mixture at pH 8.3. The characteristic sigmoidal shape of these time courses is evident under all conditions that we have investigated, with increasing concentrations of phosphorylase kinase affecting the various stages of the reaction equally (Fig. 1). We next examined the fluorescence of mixtures containing known proportions of phosphorylase a and b--at a total concentration of 2.6 mg/mL. The inset to Fig. 2 shows that the fluorescence increases linearly with the fraction of phosphorylase a, verifying that the concentration of ANS is adequate under the conditions of the assay.

The apparent lag in the time courses may reflect hysteretic activation of both phosphorylase kinase and the fragment or a unique property of the partially phosphorylated intermediate that cannot be represented by equivalent mixtures of phosphorylase a and b. Fig. 2 superimposes the reaction time courses determined in measurements of ^{32}P incorporation and of the fractional fluorescence change,

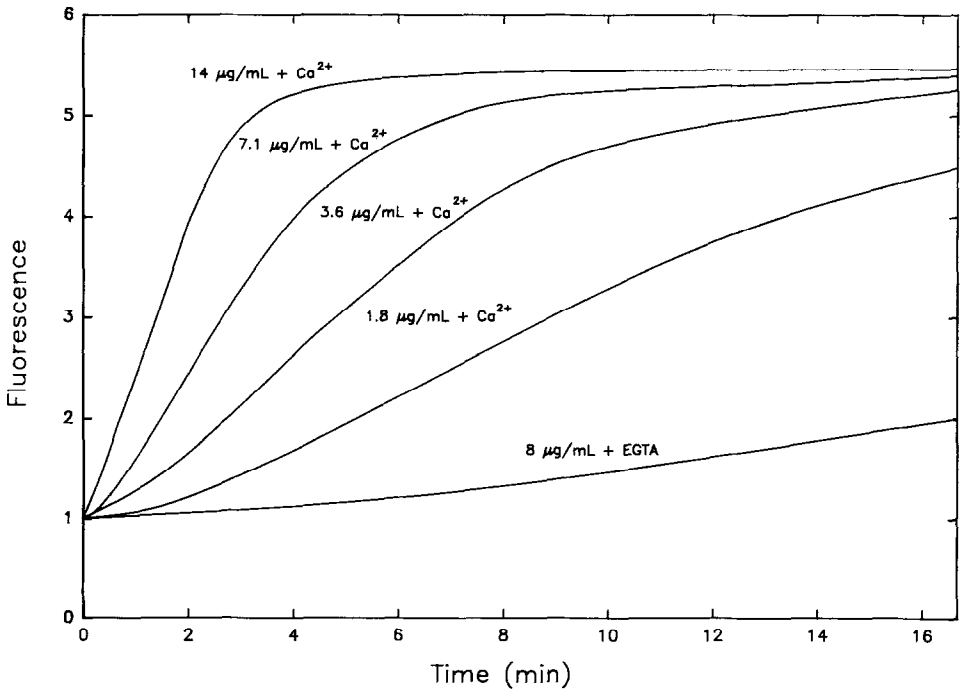

Fig. 1. Fluorescence changes initiated by the addition of varying concentrations of phosphorylase kinase to solutions containing 2.6 mg/mL phosphorylase b, 1.0 mM ATP, 6.0 mM magnesium acetate, 20 μM 1-anilinonaphthalene-8-sulfonate, 1.0 mM dithiothreitol, 50 mM Tris, 50 mM glycerophosphate, and either 0.1 mM $CaCl_2$ or 1 mM EGTA (pH 8.3, 22.5°). Excitation: 360 nm. Emission: 460 nm. Bandpass: 5 nm.

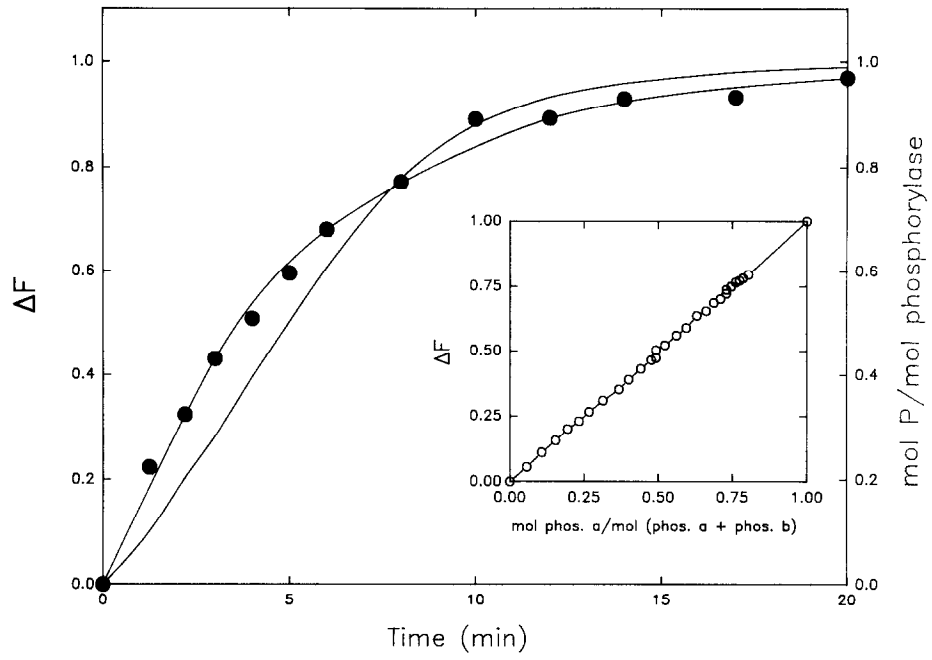

Fig. 2. Phosphorylase b to a conversion monitored in parallel measurements of ^{32}P incorporation (mol ^{32}P/92,500 g, ●) and of the relative change in fluorescence, ΔF (smooth curve). At zero time, the pure 33-kD active fragment was added to give a final concentration of 0.11 μg/mL. Conditions: as in Fig. 1 (0.1 mM $CaCl_2$, 26°). The inset shows the fluorescence of mixtures of phosphorylase a and b prepared in the assay medium (minus the active fragment).

$\Delta F = (F_{obs}-F_{phos\ b})/(F_{phos\ a}-F_{phos\ b})$. The absence of a lag period in the phosphorylation rate indicates either that the intermediate has an affinity for ANS closer to that of phosphorylase b or that its fluorescence yield is lower. We have followed the reaction at various wavelengths across the emission band, which has an uncorrected maximum at 485 nm, in order to determine whether there is a spectroscopically distinct intermediate. The results suggest that the partially phosphorylated species differs primarily in ANS binding--as do phosphorylase a and b, which have identical emission spectra (8). The fluorescence anisotropy increases slightly from 0.29 to 0.30 during the reaction.

The 5 to 6 fold increase in fluorescence intensity obtained with fresh glycerophosphate solutions is greater than that predicted from the equilibrium measurements, which were performed in 20 mM Tris ± 0.23 M KCl (8). However, the results in Table I show that enhancements of only 2 to 3 fold, depending on ionic strength, indeed occur under the conditions of the original experiments. Noting that the difference between the two buffer systems is due primarily to an effect of glycerophosphate on the phosphorylase b-ANS absorbate, we tested the effect of glucose-6-phosphate addition on the assays performed in 20 mM Tris. The result is a large increase in fluorescence enhancement, with only a ~33% reduction in the rate of reaction. In fact, the rates obtained with 20 mM Tris + 1.9 mM G-6-P and 50 mM Tris + 50 mM glycerophosphate are similar.

Table I. Effects of Assay Conditions on Fluorescence Enhancement and Rate of Reaction

[Tris] mM	[KCl] M	[G-6-P] mM	$F_{phos\ a}/F_{phos\ b}$	$d\Delta F/dt$ min^{-1}
10	0	0	2.1	.154
20	0	0	2.3	.153
10	0.2	0	3.3	.068
20	0.2	0	3.3	.067
20	0	1.9	7.8	.103
20	0.2	1.9	6.8	.052

Conditions: 0.11 μg/mL 33-kD fragment, 2.6 mg/mL phosphorylase b, 1.0 mM ATP, 6.0 mM magnesium acetate, 20 μM 1-anilinonaphthalene-8-sulfonate, 1.0 mM dithiothreitol, pH 7.3 (25°).

Although they are sigmoidal, the fluorescence time courses contain an approximately linear phase corresponding to the maximum rate of change in intensity. We have employed the maximum slope $d\Delta F/dt$ as a relative measure of the activity of the enzyme. Table II summarizes some of the catalytic properties of the chymotryptic fragment and the native enzyme that were determined in this manner. As previously reported (1), the activity of the fragment is essentially independent of calcium and of pH over the range 6.8 to 8.3. The holoenzyme, on the other hand, shows strong calcium dependence (see also Fig. 1). Activity measurements at pH 6.8 (where the fluorescence changes are similar to those found at pH 8.3) are sensitive to the increase in activity that occurs when phosphorylase kinase is phosphorylated in the reaction catalyzed by cAMP-dependent protein kinase (1,2). Table II illustrates the increased activity measured at pH 6.8 when phosphorylase kinase is pre-incubated in the assay buffer (minus phosphorylase b) with 10 μg/mL of the catalytic subunit of cAMP-dependent protein kinase and 1 mM ATP. This activity represents 44% of that measured at pH 8.3 with either the phosphorylated or non-phosphorylated enzyme. We note that the freshly

Table II. Catalytic Properties of Phosphorylase Kinase and the 33-kD Active Fragment as Determined in the Fluorometric Assay

Sample	Conc. μg/mL	$[Ca^{2+}]$ mM	pH	$d\Delta F/dt$ min^{-1}
Fragment	0.11	0	6.85	.090
	0.11	0	8.30	.095
	0.11	0.1	8.3	.086
Phos Kinase	7.1	0	8.3	<.02
	7.1	0.1	8.3	.22
	7.1	0	6.8	<.007
	7.1	0.1	6.8	.07
Activated Phos Kinase	7.1	0	6.8	<.007
	7.1	0.1	6.8	.097

Conditions: 2.6 mg/mL phosphorylase b, 1.0 mM ATP, 6.0 mM magnesium acetate, 20 μM 1-anilinonaphthalene-8-sulfonate, 1.0 mM dithiothreitol, 50 mM Tris, 50 mM glycerophosphate (23°).

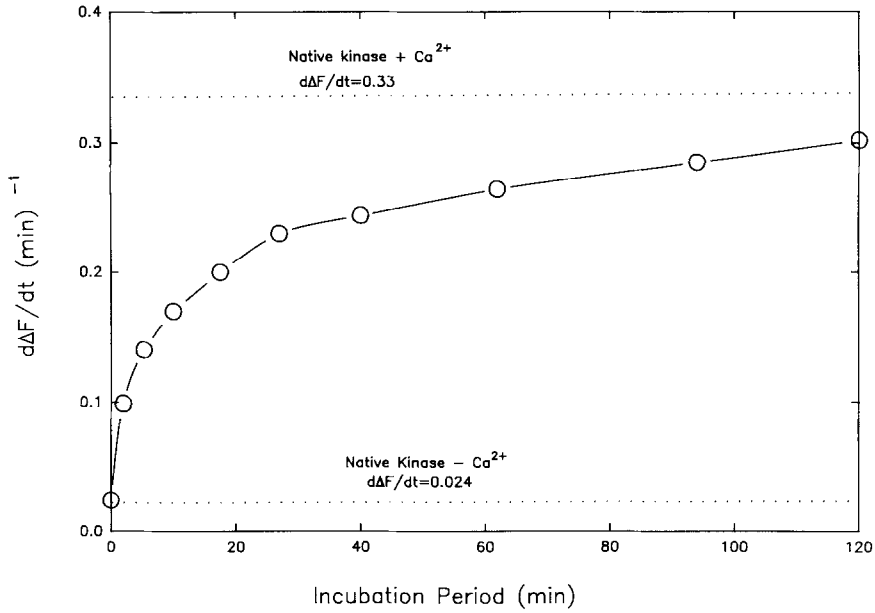

Fig. 3. Activation of phosphorylase kinase by limited chymotryptic digestion. At zero time, TLCK-treated chymotrypsin was added (7 μg/mL) to a solution containing 0.7 mg/mL phosphorylase kinase in 50 mM sodium glycerophosphate, 1 mM dithiotreitol, and 1 mM EGTA (pH 7.3, 26°). 20 μL aliquots removed at various times were assayed under the conditions shown in Fig. 1 (1 mM EGTA, 26°). The dashed lines show the activities of the undigested kinase determined in the absence (1 mM EGTA) and presence (0.1 mM) of calcium.

prepared phosphorylase kinase used in these measurements appears to have been significantly activated before treatment.

To illustrate the usefulness of this assay, we have included a time course showing the increase in calcium-independent activity that occurs when phosphorylase kinase is subjected to limited chymotryptic digestion (Fig. 3). This reaction leads to the 33-kD active fragment (1,14), which consists of three species containing residues 1-290, 1-296, and 1-298 of the γ subunit (15).

DISCUSSION

The catalytic activity of rabbit muscle phosphorylase kinase is readily determined in a continuous assay based on changes in the fluorescence of 1-anilinonaphthalene-8-sulfonate (ANS), a dye which binds preferentially to phosphorylase *a* (8). The method which we have described facilitates the rapid determination of complete time courses showing the phosphorylase *b* to *a* conversion. By using a four position cuvette compartment, four different solutions can be monitored simultaneously. Immediate information regarding activity is an advantage that expedites routine assays. For example, if a particular enzyme fraction is less active than expected, the reaction mixture can be reused directly--either for a larger aliquot or for a different sample. Maximum fluorescence changes of 5 to 8 fold are obtained in solutions containing either glycerophosphate or glucose-6-phosphate--an effector which selectively displaces

ANS from phosphorylase b (8,9,16). The presence of 20 μM ANS has no demonstrable effect on the reaction per se. No interaction of ANS with the purified chymotryptic fragment of phosphorylase kinase was detected in fluorescence intensity or anisotropy experiments (not shown).

Parallel measurements of the increases in fluorescence and of ^{32}P incorporation suggest that, in terms of ANS binding, the partially phosphorylated intermediate is more like phosphorylase b than phosphorylase a. This may relate to observations that the conformational equilibrium of the phospho-diphospho hybrid of phosphorylase is more readily shifted than that of either a or b (17). In other words, depending on conditions, the behavior of the intermediate may be close to that of either parent enzyme.

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