The quaternary structure of phosphorylase kinase as influenced by low concentrations of urea

Evidence suggesting a structural role for calmodulin

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Skeletal-muscle phosphorylase kinase is a hexadecameric oligomer composed of equivalent amounts of four different subunits, $(\alpha\beta\gamma\delta)_4$. The δ -subunit, which is calmodulin, functions as an integral subunit of the oligomer, and the γ -subunit is catalytic. To learn more about intersubunit contacts within the hexadecamer and about the roles of individual subunits, we induced partial dissociation of the holoenzyme with low concentrations of urea. In the absence of Ca^{2+} the quaternary structure of phosphorylase kinase is very sensitive to urea over a narrow concentration range. Gel-filtration chromatography in the presence of progressively increasing concentrations of urea indicates that between 1.15 M- and 1.35 M-urea the δ -subunit dissociates, allowing extensive formation of complexes larger than the native enzyme that contain equivalent amounts of α -, β - and γ -subunits. As the urea concentration is increased to 2 M and 3 M, nearly all of the enzyme aggregates to the heavy species devoid of δ -subunit. Addition of Ca^{2+} , which is known to block dissociation of the δ -subunit [Shenolikar, Cohen, Cohen, Nairn & Perry (1979) *Eur. J. Biochem.* **100**, 329–337], also blocks aggregation of the enzyme induced by the low concentrations of urea. These results suggest that in native phosphorylase kinase the δ -subunit, in addition to activating the catalytic subunit and conferring upon it Ca^{2+} -sensitivity, may also serve a structural role in preventing aggregation of the α -, β - and γ -subunits, thus limiting to four the number of $\alpha\beta\gamma\delta$ protomers that associate under standard conditions. In gel-filtration chromatography with urea a protein peak containing equivalent amounts of α - and γ -subunits is also observed, as is a peak containing only β -subunits. Increasing concentrations of urea have a biphasic effect on the activity of the holoenzyme, being stimulatory up to 1 M and then inhibitory. The concentration-dependence of urea in the inhibitory phase parallels its ability to induce dissociation of the δ -subunit.

INTRODUCTION

Phosphorylase kinase (ATP:phosphorylase-*b* phosphotransferase, EC 2.7.1.38) from rabbit skeletal muscle is a highly regulated and complex oligomeric enzyme containing a catalytic subunit and three different regulatory subunits. The catalytic subunit, γ [1], has a molecular mass of 44.7 kDa [2] and the regulatory subunits, α , β and δ , have molecular masses of 138.4 kDa [3], 125.2 kDa [4] and 16.7 kDa [5] respectively. The holoenzyme contains four copies of each of the four subunits [6] for an overall mass of 1.3×10^6 kDa [7,8]. As isolated, the holoenzyme has a low specific activity at neutrality, and is referred to as being non-activated; activation may occur through phosphorylation, among other mechanisms, of the regulatory β -subunit, and secondarily the regulatory α -subunit by cyclic AMP-dependent protein kinase [9,10].

The δ -subunit is calmodulin [11], and within the holoenzyme it has been shown to interact physically with the catalytic γ -subunit [12]. Also, a $\gamma\delta$ complex can be observed following incubation of the holoenzyme with 8 M-urea plus Ca^{2+} [12] or with 1.8 M-LiBr [13]. Moreover, the activity of free isolated γ -subunit is dramatically enhanced by calmodulin [14]. Unlike most other enzymes whose activity is regulated by calmodulin, the δ -subunit of phosphorylase kinase remains a firmly bound integral subunit of the holoenzyme even in the presence of high concentrations of the preferential Ca^{2+} -chelator EGTA [11]. The denaturant urea at 8 M has been reported to cause dissociation of the δ -subunit from phosphorylase kinase, but only in the absence of Ca^{2+} [6].

The hexadecameric holoenzyme appears to be structured as a bridged dimer of octamers, $(\alpha_2\beta_2\gamma_2\delta_2)_2$ [15]; however, inasmuch as unbridged $\alpha\gamma\delta$ trimers are observed following incubation of

the holoenzyme with LiBr [13,15], it is probable that the dimer of octamers actually subsumes a tetrameric arrangement of $\alpha\beta\gamma\delta$ protomers. In fact, however, very little is known about the subunit arrangement and interactions within the quaternary structure of the phosphorylase kinase holoenzyme. In addition to the $\gamma\delta$ and $\alpha\gamma\delta$ complexes mentioned above, a short chemical cross-linker has been used to generate $\beta\beta$ dimers from the activated, but not the non-activated, conformer of the holoenzyme [16].

In the present study we have examined whether low concentrations of a denaturant, in this case urea, can be varied to induce partial dissociation of the non-activated holoenzyme and thus yield information concerning its quaternary structure and the roles of the individual subunits. We have found that in the absence of Ca^{2+} the quaternary structure of phosphorylase kinase is very sensitive to urea over a narrow concentration range. Our data suggest that the δ -subunit, calmodulin, in addition to its regulatory role on activity, may also serve a structural role in limiting the number of protomers that associate to make-up the holoenzyme.

EXPERIMENTAL

Preparation and assay of enzymes

Non-activated phosphorylase kinase from the white skeletal muscle of female New Zealand White rabbits was isolated through to the DEAE-cellulose chromatography step [7], and was dialysed into 50 mM-Hepes/NaOH buffer, pH 6.8, containing 10% (w/v) sucrose and 0.2 mM-EDTA and then frozen until used. Its catalytic γ -subunit was prepared as described

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Fig. 1. Influence of low concentrations of urea on the quaternary structure of the holoenzyme as detected by size-exclusion chromatography
The concentrations of urea employed in generating each panel were (a) 1.0 M; (b) 1.15 M; (c) 1.35 M; (d) 1.5 M; (e) 2.0 M; (f) 3.0 M. Samples of phosphorylase kinase were diluted to 3.6 mg/ml and adjusted to the component concentrations of the appropriate dissociation buffer containing

previously [17]. Phosphorylase *b* was isolated as described by Fischer & Krebs [18], and residual AMP was removed by treatment with coconut charcoal. Bovine brain calmodulin was from Sigma Chemical Co. (P2277).

Phosphorylase kinase activity was measured by monitoring the incorporation of ^{32}P into phosphorylase *b* from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by using a filter-paper assay [17]. Specific activity of the phosphorylase kinase is expressed as μmol of phosphate transferred/min per mg of enzyme; for the holoenzyme preparations used in this study the specific activities at pH 8.2 ranged from 4.0 to 4.25 $\mu\text{mol}/\text{min}$ per mg. The activity of the isolated γ -subunit was determined as described previously [17].

Dissociation and subsequent gel filtration

By addition of stock solutions of EDTA, β -glycerophosphate, Tris and dithiothreitol, as well as urea (added either as a solid or as a stock solution), samples of phosphorylase kinase were adjusted to the same dissociation-buffer conditions against which they were subsequently dialysed. The standard dissociation buffer contained 50 mM-Tris, 50 mM- β -glycerophosphate, 1 mM-EDTA and 1 mM-dithiothreitol at pH 8.2, as well as the indicated concentrations of urea up to 8 M. Phosphorylase kinase at a final concentration of 3.6 mg/ml (Figs. 1 and 3) was dialysed against 250 ml of the dissociation buffer for 17 h and then clarified by centrifugation at 27000 *g* for 10 min. For Fig. 2 the phosphorylase kinase (2.5 mg/ml) was incubated for 17 h under the dissociation conditions, as opposed to being dialysed. A 2.0 ml portion of the kinase was then loaded on to a Bio-Gel A-5m (Bio-Rad Laboratories) column (95 cm $\times$ 1.35 cm) equilibrated with the same dissociation buffer (including urea) that was used for dialysis. The gel-filtration chromatography was carried out at 6.2 ml/h (Figs. 1 and 3) or at 4.0 ml/h (Fig. 2), and 1.0 ml fractions were collected for analyses. All of the above operations were carried out at 4 °C, except that when 8 M-urea was used the temperature was 22 °C throughout (Fig. 2*b*).

Determination of protein concentrations

The concentrations of native phosphorylase kinase and phosphorylase *b* were determined spectrophotometrically by using known absorption coefficients [7,19]. The concentrations of dissociated species of phosphorylase kinase were also estimated spectrophotometrically by using the absorption coefficient of the holoenzyme. The concentration of the isolated γ -subunit was determined with the Bio-Rad Protein Assay, with BSA as the standard, as previously described [17], and the concentrations of calmodulin solutions were based on its dry weight.

Gel electrophoresis

Protein samples were electrophoresed on SDS/polyacrylamide (10%) slab gels in the discontinuous buffer system of Laemmli [20]. The gels were stained with 0.025% Coomassie Blue G, destained with 5% (v/v) methanol/7% (v/v) acetic acid, and scanned at 595 nm with a Gelman ACD-18 automatic computing densitometer. The stoichiometry of the subunits within the new

peaks formed by the urea treatments was assessed by comparing scans of those peaks with the scan of untreated phosphorylase kinase, which was included in each electrophoresis run as an internal control.

RESULTS

Influence of urea on the structure of the holoenzyme

In the absence of Ca^{2+} the quaternary structure of phosphorylase kinase was found to be very sensitive to urea over a narrow concentration range of the denaturant. Utilizing gel-filtration chromatography, we examined the nonactivated holoenzyme's structure at concentrations of urea between 1 M and 3 M (Fig. 1). As a control, the enzyme's behaviour on the same gel-filtration column was also examined at 0 M- and 8 M-urea (Fig. 2). Protein peaks observed during chromatography were assayed for their catalytic activity at pH 8.2 and subjected to SDS/PAGE and scanning densitometry to determine their subunit composition.

The kinase was eluted as a single symmetrical peak when no urea was present (Fig. 2*a*). At 1 M- and 1.15 M-urea a portion of the protein was eluted as a broad shoulder (Figs. 1*a* and 1*b*, peak 1) before the native enzyme (Figs. 1*a* and 1*b*, peak 2). The subunit composition of the heavy shoulder (peak 1) showed equivalent amounts of α -, β - and γ -subunits, but a partial loss of the δ -subunit; this fraction also had a substantially diminished specific activity. At 1.35 M-urea (Fig. 1*c*) a substantial amount of protein was eluted before the native enzyme, and a new protein peak was eluted with the void volume (Fig. 1*c*, peak 1). Considering that the exclusion limit of the gel matrix is approx. 5×10^6 kDa, the protein in the breakthrough peak must be an aggregated form of the enzyme. It is noteworthy that, although this fraction has approximately equivalent amounts of α -, β - and γ -subunits, it has no detectable δ -subunit; and, as was the case above in the fractions that had low amounts of δ -subunit, its specific activity was very low. At concentrations of urea from 1.5 M to 3 M, very little native enzyme remained, and most of the protein was eluted in the aggregate form in the void volume, in all cases devoid of δ -subunit (Figs. 1*d*, 1*e* and 1*f*). The δ -subunit was recovered in these cases as a separate peak (peak 5). At the urea concentrations between 1.5 M and 3 M there was also a partial loss of the β -subunit (Figs. 1*d*, 1*e* and 1*f*). This subunit was recovered as a separate peak only in 8 M-urea (Fig. 2*b*, peak 3); under the other conditions it was probably lost as an insoluble precipitate. Some of the catalytically active partially dissociated species apparent in Fig. 1 were partially characterized and are described below.

It is evident that, under the conditions used to generate Fig. 1, the subunits of phosphorylase kinase, especially the δ -subunit, begin to dissociate when the concentration of urea is greater than 1 M; by 1.35 M-urea this process is very evident. Concomitant with the dissociation of the δ -subunit, there is a decrease in specific activity and an aggregation of the remaining subunits. To evaluate whether the aggregation of the enzyme at increasing

urea, and were then dialysed against that same buffer as outlined in the Experimental section. After dialysis 2 ml samples were loaded on to a Bio-Gel A-5m column equilibrated with the buffer against which the kinase had been dialysed. The gel-filtration column was developed at 6.2 ml/h, again with the same buffer. Fractions (1.0 ml) were collected and analysed for protein by their absorbance at 280 nm. Those fractions indicated by the numbered arrows were then assayed for their subunit composition by SDS/PAGE and for their catalytic activity at pH 8.2, giving the values shown in the last five columns of the Figure. Activity assays were initiated by adding 10 μl of each fraction to 90 μl of an assay mixture containing all the components necessary for phosphorylase *b* conversion. The final concentrations in the assay mixture at pH 8.2 were 50 mM-Tris/HCl, 50 mM- β -glycerophosphate, 0.1 mM-dithiothreitol, 0.1 mM-EGTA, 10 mM-magnesium acetate, 0.27 mM- CaCl_2 , 1.5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 3.0 mg of phosphorylase *b*/ml. After 6 min at 30 °C a sample was removed to determine ^{32}P incorporation into phosphorylase. Lane C in the SDS/PAGE gels (central column) refers to control phosphorylase kinase holoenzyme included as an internal standard. The 'N.D.' in the 'Specific activity' column signifies that activity was not detectable in those particular fractions. The common non-activated phosphorylase kinase used in generating all six panels of the Figure was obtained by pooling three different enzyme preparations.

Fig. 2. Influence of 8 M-urea on the quaternary structure of the holoenzyme

Samples of phosphorylase kinase were adjusted to 2.5 mg/ml and the component concentrations of the dissociation buffer, which either did (panel b) or did not (panel a) contain 8 M-urea. After incubation for 17 h 2.0 ml samples were applied to the gel-filtration column and chromatographed at 4 ml/h. The remainder of the procedures and the description of the Figure are the same as described in the legend to Fig. 1. The specific activity of the control enzyme (peak 1 in panel a) was 4.2 μ mol of phosphate transferred/min per mg following incubation and chromatography, i.e. the same as the untreated native enzyme (see the Experimental section). A single, separate, preparation of non-activated phosphorylase kinase was used in generating the two panels of this Figure.

concentrations of urea might be caused by dissociation of the δ -subunit, we examined urea's influence on structure in the presence of Ca^{2+} , which has previously been shown to inhibit dissociation of the δ -subunit in the presence of 8 M-urea [6]. If dissociation of δ -subunit is associated with aggregation, then one would predict that there would be significantly less aggregated protein if the experiment shown in Fig. 1 were carried out in the presence of Ca^{2+} . That this is in fact the case is shown in Fig. 3. In this experiment three samples of phosphorylase kinase were dialysed against 1.4 M-urea in the presence and in the absence of 2 mM free Ca^{2+} . Before dialysis, excess calmodulin was added to one of the samples exposed to Ca^{2+} . The samples were then passed through gel-filtration columns equilibrated with the buffer used for dialysis. As previously, protein peaks were assayed and analysed by SDS/PAGE. In the presence of Ca^{2+} (Fig. 3b) nearly all of the protein was eluted in the peak of native enzyme, and there was virtually no aggregated protein present in the void volume; however, as before, an aggregate was observed in the absence of Ca^{2+} (Fig. 3a, peak 1). Inclusion of calmodulin with the Ca^{2+} caused little change in the elution pattern from that observed with Ca^{2+} alone, except for the occurrence of a relatively large peak that was lighter than the holoenzyme and contained all four subunits (Fig. 3c, peak 3).

Influence of urea on catalytic activity

Because other reagents that might be considered as general structural perturbants have been shown, at low concentrations, to enhance the activity of phosphorylase kinase [21–23], we decided to examine directly the influence of urea on the activity

of the holoenzyme. This was done in two different ways: first, by preincubating the enzyme with urea, as in Figs. 1–3, and then diluting out the denaturant before catalytic assays, and, secondly, by adding urea directly to the assays without prior incubation of the kinase with the denaturant.

The effect of treatment with urea under the first condition is shown in Fig. 4. Here the holoenzyme was incubated with the indicated concentrations of urea at 4 °C for 17 h in the presence and in the absence of Ca^{2+} . Following this incubation, the enzyme (and urea) was diluted 1000-fold into an activity-assay mixture. Progressively increasing concentrations of urea had a biphasic effect on activity, first stimulatory and then inhibitory (Fig. 4). The maximal stimulation occurred at 1 M-urea, and increasing the urea concentration past that diminished activity. At all concentrations of urea preincubation in the presence of Ca^{2+} resulted in greater activity, which was most pronounced at concentrations of urea greater than 1 M. These results, considered together with those of Figs. 1 and 3, are consistent with the inactivation of the holoenzyme by concentrations of urea above 1 M being caused, at least in part, by weakened interactions with the δ -subunit. At concentrations of urea up to 1 M, the enzymic activity progressively increased. The probable explanation for this stimulation (see the Discussion section) is that interactions between the catalytic γ -subunit and the regulatory α - and/or β -subunits become perturbed, thus weakening the constraints on catalytic activity imposed by the non-activated holoenzyme's quaternary structure [17,24].

A similar biphasic effect of urea, again with a peak at 1 M, was observed when it was included directly in activity assays without

Fig. 3. Inhibition by Ca^{2+} of dissociation induced by 1.4 M-urea

Samples of phosphorylase kinase were adjusted to 3.6 mg/ml and the component concentrations of the dissociation buffer, dialysed and chromatographed against dissociation buffer containing 1.4 M-urea. In panels (b) and (c) the dissociation buffer also contained 3 mM- CaCl_2 . In addition, calmodulin at a final concentration of 150 $\mu\text{g}/\text{ml}$ was added to the kinase used to generate panel (c) before the dialysis; however, it was not included in the dissociation buffer. The remainder of the procedures and the description of the Figure are the same as described in the legend to Fig. 1. A single, separate, preparation of non-activated phosphorylase kinase was used in generating the three panels of this Figure.

prior incubation with the kinase (Fig. 5a). The maximal stimulation at 1 M-urea was 14-fold, whereas by 3 M-urea the activity was less than the control with no denaturant. To evaluate whether the stimulatory effect of urea was due to perturbation of subunit interactions within the holoenzyme or to a direct influence of urea on the catalytic γ -subunit, including its interaction with phosphorylase *b*, we assayed the activity of the isolated γ -subunit in the presence of increasing concentrations of urea (Fig. 5b). No activation was observed; instead, the activity progressively decreased as the concentration of urea was increased. These results suggest that the stimulation of the holoenzyme's activity by urea is not due to a direct influence of the denaturant on the structure of the catalytic subunit, nor is it due to a perturbation of the structure of phosphorylase *b* to make it a better substrate.

Partial characterization of new species induced by urea

Those new protein peaks containing approximately equivalent amounts of different subunits produced by the various concentrations of urea shown in Fig. 1 were further characterized. The protein that was eluted at the void volume of the gel-filtration column after incubation of phosphorylase kinase with

1.35 M-urea contained approximately equal amounts of α -, β - and γ -subunits (Fig. 1c, peak 1). At higher concentrations of urea the amount of α -subunit present in this peak diminished; thus the peak produced by 1.35 M-urea was studied. This protein peak, which must represent an aggregated form of the enzyme, retained activity, although its specific activity was only a few per cent of that of the native enzyme (see the Experimental section). The activity in this peak was not diminished by addition of EGTA at a concentration exceeding that of Ca^{2+} , nor was it stimulated by the addition of exogenous Ca^{2+} /calmodulin (results not shown).

In the presence of 2 M-urea a late-eluted peak was observed that contained equivalent amounts of α - and γ -subunits (Fig. 1e, peak 4). This peak, like that of the one above containing α -, β - and γ -subunits, had a low specific activity, was insensitive to EGTA and was not stimulated by addition of Ca^{2+} /calmodulin.

When the kinase was incubated with excess calmodulin and dissociated with 1.4 M-urea in the presence of Ca^{2+} , a peak was observed that had the subunit composition of approximately two δ -subunits (calmodulin) for each α -, β - and γ -subunit (Fig. 3c, peak 3). This peak, which was eluted well after the native

Fig. 4. Effect of Ca^{2+} on the inactivation by urea of the holoenzyme

Phosphorylase kinase (at 0.5 mg/ml) was incubated at 4 °C for 17 h in a mixture at pH 8.2 containing (final concentrations) 50 mM-Tris/HCl, 50 mM- β -glycerophosphate, 0.1 mM-EDTA, 0.1 mM-dithiothreitol and the indicated concentrations of urea in the absence (●) and in the presence (○) of 0.3 mM- CaCl_2 . Following this incubation the enzyme was diluted 100-fold with a pH 6.8 dilution buffer that contained 50 mM-Tris/HCl, 50 mM- β -glycerophosphate, 0.1 mM-EDTA and 0.1 mM-dithiothreitol. A portion of this diluted enzyme was then diluted an additional 10-fold into an assay mixture to initiate the phosphorylase *b* conversion assays at 30 °C and pH 6.8. Final concentrations in the assay mixture were 50 mM-Tris/HCl, 50 mM- β -glycerophosphate, 0.1 mM-EGTA, 0.1 mM-dithiothreitol, 0.3 mM- CaCl_2 , 1.5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 10 mM-magnesium acetate, 3.0 mg of phosphorylase *b*/ml and 0.5 μg of phosphorylase kinase/ml. After 10 min a sample was removed from the assay mixture for determination of ^{32}P incorporation into phosphorylase. Note that the preincubation concentrations of urea indicated on the abscissa were diluted 1000-fold into the assay and that all assay mixtures contained a fixed amount of Ca^{2+} regardless of whether it was present in or absent from the preincubations with urea before the assays.

enzyme, had a high specific activity equivalent to that of the peak of holoenzyme eluted under these dissociation conditions (compare right-hand column of Fig. 3c for peaks 2 and 3).

DISCUSSION

Non-phosphorylated, i.e. non-activated, phosphorylase kinase holoenzyme has a very low phosphotransferase activity at neutral pH (for a review see ref. [9]). It has been suggested that this is because the activity of the non-activated enzyme is under severe quaternary constraint at this pH [7,25]. That is to say, the activity of the catalytic subunit is repressed by its interactions with other subunits within the non-activated holoenzyme. Inasmuch as calmodulin, the δ -subunit, is known to stimulate the activity of the isolated catalytic γ -subunit [14], the proposed inhibitory interactions would be expected to come from the α - or β -subunits. In fact, an equimolar mixture of α - and β -subunits has been shown to inhibit the catalytic activity of the γ -subunit and the $\gamma\delta$ complex effectively [17]. Because of this idea that there is a quaternary constraint on activity imposed by the α - and/or the β -subunit, it has been suggested that most perturbations in quaternary structure will result in activation of the non-activated enzyme [16]. Thus it is not surprising to find that the enzyme's activity is stimulated by such diverse agents as neutral salts at high concentrations [21], organic solvents [22] and phospholipids [23]. The data in Figs. 4 and 5(a) indicate that urea can also be thought of as a structural perturbant that activates. When urea

Fig. 5. Effect of inclusion of urea in the catalytic assays on the phosphorylase-conversion activities of the holoenzyme and of the isolated catalytic subunit

(a) Non-activated phosphorylase kinase at 0.1 $\mu\text{g}/\text{ml}$ was incubated at pH 6.8 and 30 °C in a mixture containing (final concentrations) 50 mM-Tris/HCl, 50 mM- β -glycerophosphate, 90 μM -EGTA, 90 μM -dithiothreitol, 0.3 mM- CaCl_2 , 1.5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 10 mM-magnesium acetate, 4.5 mg of phosphorylase *b*/ml and the indicated concentrations of urea. After 14 min samples were removed for determination of ^{32}P incorporation into phosphorylase. (b) The isolated catalytic γ -subunit prepared exactly as described in ref. [17] was diluted 10-fold with pH 6.8 buffer containing (final concentrations) 50 mM-Tris/HCl, 50 mM- β -glycerophosphate, 0.1 mM-EGTA and 0.1 mM-dithiothreitol. The diluted catalytic γ -subunit was then diluted an additional 11-fold into an assay mixture at pH 6.8 that contained all components of the activity assay except MgATP and the varied urea and was allowed to renature for 15 min at room temperature. After addition of the various concentrations of urea, reactions were immediately initiated by addition of MgATP. The final concentrations in the assay mixture were 50 mM-Tris/HCl, 50 mM- β -glycerophosphate, 0.1 mM-EGTA, 0.1 mM-dithiothreitol, 1.5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 10 mM-magnesium acetate, 2 mg of phosphorylase *b*/ml, 0.66 μg of catalytic γ -subunit/ml and the indicated concentrations of urea. Note that, in addition to the added concentrations of urea indicated on the abscissa, all assay mixtures also contained 73 mM carry-over urea from preparation of the γ -subunit. After 6 h at 30 °C samples were removed from the assay mixture for determination of ^{32}P incorporation into phosphorylase.

is included directly in the assay mixtures a maximal stimulation of 14-fold occurs at 1 M (Fig. 5a). It should be noted, however, that the observed stimulation of the holoenzyme by urea, presumably caused by perturbation of the quaternary structure, would probably be much greater if the urea did not simultaneously inhibit the phosphotransferase activity of the catalytic subunit, as shown in Fig. 5(b). In fact, if the 80% inhibition of phosphotransferase activity by 1 M-urea observed in Fig. 5(b) with the isolated γ -subunit also occurs with the γ -subunits within the holoenzyme, then the overall maximal stimulation determined in Fig. 5(a) would need to be 70-fold in order to actually observe a net stimulation of 14-fold. If this premise is correct, it would imply that the denaturant urea might be as effective an activator of the enzyme as is phosphorylation.

As was pointed out in the Introduction, the δ -subunit (calmodulin) interacts with the γ -subunit within the holoenzyme and also profoundly stimulates the catalytic activity of the isolated γ -subunit. Consistent with this stimulatory role of calmodulin on activity, all the peak fractions in Figs. 1 and 3 that contained γ -subunit, but had low amounts of δ -subunit, also had low specific activities. From comparison of Figs. 4 and 1 it seems probable that some, or perhaps even most, of the inactivation that occurs when the holoenzyme is incubated in the absence of Ca^{2+} with concentrations of urea exceeding 1 M is due to

dissociation of calmodulin from the enzyme, with a resultant loss in activity. It is difficult to say if this reasoning also applies to the inhibitory portion of the activation curve in Fig. 5(a), where high concentrations of urea are directly included in the assays because of the presence in those assays of Ca^{2+} , which should block dissociation of the calmodulin. Perhaps the lower concentration of enzyme in the assays or the presence of other assay components allows dissociation of, or weakened interactions with, δ -subunit resulting in a decrease in activity. Alternatively, as the concentration of urea is increased above 1 M its inhibitory influence on the phosphotransferase activity of the catalytic subunit, as shown in Fig. 5(b), may overcome its stimulatory influence on the holoenzyme's activity caused by perturbation of quaternary structure. At this time we simply do not know the actual mechanism responsible for the decrease in activity that occurs when concentrations of urea in the assay mixtures exceed 1 M (Fig. 5a).

In addition to its role of activating the catalytic subunit, and in the process conferring on it Ca^{2+} -sensitivity, the data in Figs. 1 and 3 suggest that calmodulin may also have a structural role within the oligomeric phosphorylase kinase holoenzyme. As the concentration of urea was progressively increased over a narrow range, the δ -subunit dissociated and the remaining subunits aggregated (Fig. 1); however, when Ca^{2+} was included to block dissociation of the δ -subunit aggregation did not occur (Fig. 3). These data suggest that aggregation is caused by the dissociation of calmodulin induced by the low concentrations of urea, as opposed to being a direct result of the urea itself. Additional evidence supporting the hypothesis that dissociation of the δ -subunit leads to aggregation comes from a previous investigation that examined the effects of heparin on the structure of the holoenzyme [26]. In that report it was noted that addition of heparin to nonactivated phosphorylase kinase at pH 7 in the absence of Ca^{2+} caused formation of heavy aggregates that contained significantly less δ -subunit than the native enzyme. (Perhaps heparin as a polyanion displaces the acidic calmodulin subunit.) Hinrichsen *et al.* [27] have recently reconsidered these earlier observations on the effect of heparin and also concluded that the δ -subunit may be blocking the sites responsible for aggregation. Thus two completely different agents, urea and heparin, cause formation of aggregates diminished in δ -subunit. The tendency for protomers devoid of the δ -subunit to aggregate could possibly explain why phosphorylase kinase has evolved with the unusual property for a calmodulin-dependent enzyme of having calmodulin as an integral subunit, regardless of the Ca^{2+} concentration. Perhaps the addition of δ -subunit to the $\alpha\beta\gamma$ complex may, under standard conditions, create an association geometry that limits the number of protomers that associate to four. One cannot help but wonder how many other high-affinity Ca^{2+} -independent interactions between calmodulin and target proteins (for a listing see ref. [28]) may also connote a structural role for calmodulin.

It must be pointed out that phosphorylase kinase may also aggregate under conditions that would not be expected to cause dissociation of the δ -subunit. For instance, Ca^{2+} and Mg^{2+} act synergistically to cause a reversible aggregation of the non-activated holoenzyme [29]. One would not expect the δ -subunit to dissociate under these conditions, and in fact no diminution in the relative amount of δ -subunit in the aggregate has been observed (D. F. Hamill & G. M. Carlson, unpublished work). A

small amount of aggregated phosphorylase kinase is also present in the freshly purified enzyme, and this amount has been reported to increase with storage [7]; however, the driving force for this aggregation and the subunit composition of the aggregates are not known. Thus, although our data suggest that dissociation of δ -subunit leads to aggregation of phosphorylase kinase, it is probable that under other conditions aggregation may also occur through a separate mechanism that is not dependent on dissociation of the δ -subunit.

This work was supported by National Institutes of Health Grant DK 32953.

REFERENCES

- Skuster, J. R., Chan, K.-F. J. & Graves, D. J. (1980) *J. Biol. Chem.* **255**, 2203–2210
- Reimann, E. R., Titani, K., Ericsson, L. H., Wade, R. D., Fischer, E. H. & Walsh, K. A. (1984) *Biochemistry* **23**, 4185–4192
- Zander, N. F., Meyer, H. E., Hoffmann-Posorske, E., Crabb, J. W., Heilmeyer, L. M. G., Jr. & Kilimann, M. W. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2929–2933
- Kilimann, M. W., Zander, N. F., Kuhn, C. C., Crabb, J. W., Meyer, H. E. & Heilmeyer, L. M. G., Jr. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9381–9385
- Grand, R. J. A., Shenolikar, S. & Cohen, P. (1981) *Eur. J. Biochem.* **113**, 359–367
- Shenolikar, S., Cohen, P. T. W., Cohen, P., Nairn, A. C. & Perry, S. V. (1979) *Eur. J. Biochem.* **100**, 329–337
- Cohen, P. (1973) *Eur. J. Biochem.* **34**, 1–14
- Hayakawa, T., Perkins, J. P., Walsh, D. A. & Krebs, E. G. (1973) *Biochemistry* **12**, 567–573
- Pickett-Gies, C. A. & Walsh, D. A. (1986) *Enzymes* 3rd Ed. **17**, 395–459
- Ramachandran, C., Goris, J., Waelkens, E., Merlevede, W. & Walsh, D. A. (1987) *J. Biol. Chem.* **262**, 3210–3218
- Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. C. & Nairn, A. C. (1978) *FEBS Lett.* **92**, 287–293
- Picton, C., Klee, C. B. & Cohen, P. (1980) *Eur. J. Biochem.* **111**, 553–561
- Chan, K. F. J. & Graves, D. J. (1982) *J. Biol. Chem.* **257**, 5939–5947
- Kee, S. M. & Graves, D. J. (1986) *J. Biol. Chem.* **261**, 4732–4737
- Trempe, M. R., Carlson, G. M., Hainfeld, J. F., Furciniti, P. S. & Wall, J. S. (1986) *J. Biol. Chem.* **261**, 2882–2889
- Fitzgerald, T. J. & Carlson, G. M. (1984) *J. Biol. Chem.* **259**, 3266–3274
- Paudel, H. K. & Carlson, G. M. (1987) *J. Biol. Chem.* **262**, 11912–11915
- Fischer, E. H. & Krebs, E. G. (1958) *J. Biol. Chem.* **231**, 65–71
- Kastenschmidt, L. L., Kastenschmidt, J. & Helmreich, E. (1968) *Biochemistry* **7**, 3590–3608
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Carlson, G. M. & Graves, D. J. (1976) *Biochemistry* **15**, 4476–4481
- Singh, T. J. & Wang, J. H. (1979) *J. Biol. Chem.* **254**, 8466–8472
- Kyriakidis, S. M., Sotiroidis, T. G. & Evangelopoulos, A. E. (1986) *Biochem. Int.* **13**, 853–861
- Paudel, H. K. & Carlson, G. M. (1988) *Arch. Biochem. Biophys.* **264**, 641–646
- Hayakawa, T., Perkins, J. P. & Krebs, E. G. (1973) *Biochemistry* **12**, 574–580
- Hessova, A., Varsanyi, M. & Heilmeyer, L. M. G., Jr. (1985) *Eur. J. Biochem.* **146**, 107–115
- Hinrichsen, A., Bockl, H. J. & Schramm, H. J. (1989) *Biol. Chem. Hoppe-Seyler* **370**, 125–133
- Hooper, J. E. & Kelly, R. B. (1984) *J. Biol. Chem.* **259**, 148–153
- Carlson, G. M. & King, M. M. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 869