

PHOSPHOCREATINE REPRESENTS A THERMODYNAMIC AND FUNCTIONAL IMPROVEMENT OVER OTHER MUSCLE PHOSPHAGENS

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Summary

In vertebrate tissues, the only phosphagen is phosphocreatine (PC), and the corresponding phosphotransferase is creatine phosphokinase (CPK). Among invertebrates, a variety of phosphotransferase reactions are found in addition to CPK, including arginine phosphokinase (APK), glycyamine phosphokinase (GPK), taurocyamine phosphokinase (TPK) and lombricine phosphokinase (LPK). Although there is some uncertainty about the exact value, the apparent equilibrium constant for the CPK reaction ($K'_{\text{cpk}} = [\text{creatine}][\text{ATP}]/[\text{PC}][\text{ADP}]$), under physiological conditions similar to those of vertebrate muscle, ranges from 100 to 160. The corresponding K' value for the APK reaction is somewhat controversial, and K' values for the GPK, TPK and LPK reactions are not known. In this study, conventional and ^{31}P -NMR methods were used to evaluate the equilibrium constants for the APK, GPK, TPK and LPK reactions relative to that of CPK. The corresponding K' values for the APK, GPK, TPK and LPK reactions, expressed as a percentage of K'_{cpk} , are 13, 29, 29 and 32 %, respectively. The exclusively invertebrate phosphagens exist as a cohort of thermodynamically more stable compounds. Thus, PC constitutes a thermodynamic (and functional) improvement, in that the CPK reaction is able to buffer ATP at much higher ATP/ADP ratios than are other phosphagens. However, possession of a phosphagen system with a lower K' value may be advantageous under certain specific physiological conditions such as intracellular acidosis.

Introduction

Phosphocreatine (PC) (structure shown in Fig. 1) and the corresponding transphosphorylation enzyme, creatine phosphokinase (CPK) ($\text{PC} + \text{ADP} \leftrightarrow \text{creatine} + \text{ATP}$), together represent the only phosphagen system in vertebrates. The apparent equilibrium constant ($K'_{\text{cpk}} = [\text{creatine}][\text{ATP}]/[\text{PC}][\text{ADP}]$) under physiological conditions ranges from 100 to 160 (Lawson & Veech, 1979). In vertebrate striated skeletal muscle, PC concentrations are 3–4 times higher than ATP concentrations and 2.5 times higher than free creatine concentrations (Meyer *et al.* 1985). Furthermore, free ADP (ADP_{free}) concentrations are in the micro-

Key words: phosphagen, phosphokinase, phosphocreatine, muscles.

PC in intracellular energy transport *via* the phosphocreatine shuttle has been proposed by Bessman and coworkers (Bessman & Geiger, 1981; Bessman, 1987). This hypothesis has generated a considerable amount of controversy. Meyer *et al.* (1984) have recently shown that the proposed transport function of PC is a direct result of the ATP buffering role of the CPK reaction. In fact, they refer to the transport function as spatial ATP buffering (Meyer *et al.* 1984). An additional role of CPK in the regulation of glycolysis has been suggested (Davuluri *et al.* 1981). Pi levels are typically quite low in fast twitch muscles (Meyer *et al.* 1985). Release of Pi by PC utilization appears to be a major prerequisite for maximum activation of glycolysis, as Pi is a substrate for glycogen phosphorylase (Meyer *et al.* 1986). All the above proposed functions of PC in muscles stem from the high K' for the CPK reaction, strongly in favour of PC breakdown.

Around the time of discovery of PC, Meyerhof & Lohmann (1928) found an acid-labile compound in crayfish muscle that was shown to be phosphoarginine (PA) (Fig. 1). An extensive survey of the distribution of PC and PA showed that PA was not present in vertebrate muscles, suggesting that PA was the 'invertebrate' phosphagen (Meyerhof, 1930). However, further critical studies showed that PC was present in certain polychaetes (Needham *et al.* 1932) and in echinoderms (Yudkin, 1954). Starting in the early 1950s, Van Thoai and coworkers in Paris discovered a striking new array of major phosphagens in worms (reviewed by Van Thoai & Robin, 1969). These phosphagen acceptors are all guanidine derivatives (Fig. 1). Polychaetes as a group have six *major* phosphagens – PC, PA, phosphoglycocyamine (PG), phosphotaurocyamine (PT), phosphohypotaurocyamine (PHT) and phospholombricine (PL). Phosphagen type may differ among species or among tissues within a given species (Robin, 1974). The distribution of phosphagens in the polychaetes generally correlates well with the systematics of the group (Robin, 1974). Oligochaetes have PL, whereas sipunculids have PHT in their muscles except for one species which has PA (Van Thoai & Robin, 1969).

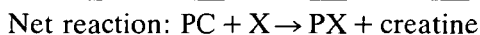
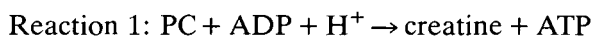
Considerable attention has been focused on the evolution of phosphagen systems in the animal kingdom. CPK, arginine phosphokinase (APK), glycocyamine kinase (GPK), taurocyamine kinase (TPK), hypotaurocyamine kinase (HTPK) and lombricine kinase (LPK) are all homologous, presumably having originated from the ancestral APK gene (Van Thoai, 1968; Watts, 1968, 1971, 1975). Thus, PA is often regarded as the most primitive of the phosphagens. It has been suggested that the presence of PC in higher animals (the vertebrates?) represents a functional improvement over PA because creatine is a dead-end compound and is therefore isolated from amino acid metabolism (Watts, 1975). This explanation provides a convenient rationale for the use of PC rather than PA, but certainly does not help us to explain the presence of PG, PT, PHT and PL in the animal kingdom. Robin (1974) suggests that the evolution of GPK, TPK, HTPK and LPK 'may reflect special attempts to find out alternative phosphagens more fitted to their biological function than PC and PA [or]... metabolic imperatives particular to this group of invertebrates'. In the case of PG, Watts (1971) has suggested that the lack of a methyl group on glycocyamine makes this

substrate more symmetrical and less able to induce the enzyme to catalyse the transphosphorylation reaction.

Given the importance of the high K'_{cpk} in terms of the functional roles of the CPK reaction, it is rather surprising that potential differences in the thermodynamic properties of the phosphagen phosphotransferase reactions have not been systematically considered as selective factors in the evolution of phosphagen systems in the animal kingdom. The exact value of K'_{apk} is somewhat controversial. It is clear, however, that K'_{apk} is considerably less than K'_{cpk} (Morrison, 1973; Rao *et al.* 1976; Graham *et al.* 1986). The thermodynamic properties of the GPK, TPK, HTPK and LPK reactions are not known. In the present study, the apparent equilibrium constants for four of the major invertebrate phosphagen phosphotransferase reactions, relative to that of CPK, have been determined. The results show that the values for K'_{apk} , K'_{gpk} , K'_{tpk} and K'_{lpk} are 70–90 % lower than that of K'_{cpk} , indicating that the invertebrate phosphagens exist as a cohort of thermodynamically more stable compounds.

Determination of the thermodynamic properties of phosphagen phosphotransferase reactions: theory

The determination of the thermodynamic properties of phosphagen phosphotransferase reactions is complicated by the superimposed equilibria of Mg^{2+} and the adenylates (Lawson & Veech, 1979). Furthermore, these reactions involve consumption or production of protons (Pörtner *et al.* 1984). Walker and coworkers (Annesley & Walker, 1977; Turner & Walker, 1985) developed a method for evaluating the properties of phosphorylated analogues of creatine relative to PC. A reaction mixture consisting of PC, the creatine analogue (X), ATP and CPK is allowed to reach equilibrium. Two reactions take place – PC conversion to creatine (reaction 1) and analogue phosphorylation (reaction 2).



where PX is the phosphorylated analogue. The net reaction shows that protons and the adenylates cancel out. The K' values for each component reaction are derived from the following relationships:

$$K'_{\text{cpk}} = ([\text{creatine}][\text{ATP}])/([\text{PC}][\text{ADP}]),$$

$$K'_{\text{xpk}} = ([\text{X}][\text{ATP}])/([\text{PX}][\text{ADP}]).$$

Since both reactions share ADP and ATP, the equations can be rearranged as follows:

$$[\text{creatine}]/([\text{PC}]K'_{\text{cpk}}) = [\text{X}]/([\text{PX}]K'_{\text{xpk}})$$

or

$$K'_{\text{cpk}}/K'_{\text{xpk}} = ([\text{PX}][\text{creatine}])/([\text{PC}][\text{X}]).$$

Thus, if the equilibrium concentrations of PC, creatine, PX and X are known, it is possible to calculate the combined equilibrium constant for the reaction ($K'_{\text{comb}} = K'_{\text{cpk}}/K'_{\text{xpk}}$). The reciprocal of K'_{comb} ($1/K'_{\text{comb}} = K'_{\text{xpk}}/K'_{\text{cpk}}$) is useful in comparing the thermodynamic properties of XPK with CPK reactions. A value for $1/K'_{\text{comb}}$ of less than 1.0 indicates that the phosphorylated creatine analogue is thermodynamically more stable than PC. This approach is valid only if the pK'_a values for the phosphate groups of PC and PX are very close to each other. This appears to be a reasonable assumption since the pK_a for this group in both PC and PA is 4.5 (Jenks & Regenstein, 1973).

In the present study, this approach was utilized to evaluate the thermodynamic properties of the APK, GPK, TPK and LPK reactions, relative to CPK, by using reaction mixtures consisting of PC, CPK, ATP, phosphate acceptor (L-arginine, glycocyamine, taurocyamine or lombricine) and the appropriate phosphotransferase (APK, GPK, TPK or LPK). Equilibrium concentrations were measured by standard enzyme-linked, spectrophotometric assays (described in Materials and methods). In addition, phosphorus nuclear magnetic resonance (^{31}P -NMR) spectroscopy was used as an independent method for evaluating equilibrium concentrations. ^{31}P -NMR spectra yield quantitative data on the relative concentrations of PC and PX in the reaction mixtures but provide no information about concentrations of creatine and X. However, if the initial PC concentration is equal to the concentration of X (and no PX or creatine is present initially), then the final creatine and concentrations will be equal to the final PX and PC concentrations, respectively. Therefore, by substitution, the following relationship is obtained:

$$1/K'_{\text{comb}} = K'_{\text{xpk}}/K'_{\text{cpk}} = ([\text{PC}][\text{PC}])/([\text{PX}][\text{PX}]) = ([\text{PC}]/[\text{PX}])^2.$$

Thus, it is possible to estimate K'_{comb} simply by taking the square of the ratio of the integrals of PC over PX in spectra of equilibrium mixtures when the initial concentrations of PC and X are equal (details of NMR spectroscopy are discussed in Materials and methods).

Materials and methods

Materials

GPK, TPK and LPK were purified from the body wall musculature of the annelids *Nereis virens* (polychaete), *Arenicola cristata* (polychaete) and *Lumbricus terrestris* (earthworm), respectively. These phosphotransferases were purified according to the generalized protocol of Hoffmann (1981) utilizing ammonium sulphate fractionation, DEAE-Sephacel ion-exchange chromatography and hydroxylapatite chromatography steps. An additional gel filtration step (Sephadex G-100) was necessary for purification of LPK. Specimens of *N. virens* were obtained from the Marine Biological Laboratory (Woods Hole, Massachusetts, USA). Carolina Biological (Burlington, North Carolina, USA) was the commercial source of *L. terrestris*. Specimens of *A. cristata* were collected intertidally in Tampa Bay, Florida, USA. All muscle samples were blotted free of internal

organs, frozen in liquid nitrogen and stored at -70°C prior to enzyme purification. APK was purchased from Sigma Chemical Co. (St Louis, Missouri, USA). This enzyme was further purified by hydroxylapatite chromatography. CPK was from Boehringer Mannheim Biochemicals. All phosphotransferase preparations were free of myokinase, ATPase and phosphatase activities. All phosphotransferase preparations proved unstable, showing substantial deterioration of activity over several weeks even in the presence of 2-mercaptoethanol.

Taurocyamine was synthesized from thiourea, taurine and ethyl bromide according to the protocol of Brand & Brand (1942) for the synthesis of glycocyamine. Structure and purity were validated by natural abundance ^{13}C -NMR spectroscopy, thin layer chromatography (TLC) and by direct enzymatic assay (Lowry & Passonneau, 1972) using TPK substituted for CPK in the assay for creatine.

Lombricine was purified as a natural product from whole *L. terrestris* as described by Hoffmann (1981) with some modifications. Approximately 1 kg of freshly thawed worms was homogenized in 2 l of distilled water using a Waring blender. After centrifugation (20 min, 10 000 g), 70 % perchloric acid was added slowly to the supernatant to achieve a final concentration of 6 %. This was followed by centrifugation (20 min, 10 000 g). The supernatant was heated to 75°C for 15 min, allowed to cool and then centrifuged (20 min, 10 000 g). The supernatant was brought to pH 5.5–6.0 with solid K_2CO_3 , allowed to sit for 15 min and then centrifuged (20 min, 10 000 g). The resulting supernatant was brought to pH 1.8 with HCl and applied to a Dowex-50 (H^+) column (35 cm \times 4.5 cm, 600 ml). The column was exhaustively washed with 0.01 mol l^{-1} HCl. Amino acids and related compounds were eluted with 2 mol l^{-1} NH_4OH . Small volumes of each fraction were spotted on cellulose TLC plates and run in a solvent system consisting of pyridine, ethanol, NH_4OH and water (3:3:3:1). Guanidine compounds, visualized using Sakeguchi's reagent (Pant, 1959), were pooled and lyophilized. The residue was dissolved in 25 ml of 40 mmol l^{-1} pyridine and 400 mmol l^{-1} formic acid (pH 2.6) and applied to a Dowex-50 (pyridine) column (35 cm \times 2.7 cm, 223 ml) which had been equilibrated with 40 mmol l^{-1} pyridine and 400 mmol l^{-1} formic acid (pH 2.6). The column was washed with 700 ml of equilibration buffer followed by 1 l of 100 mmol l^{-1} pyridine and 300 mmol l^{-1} formic acid (pH 3.1). Fractions, identified by TLC as containing lombricine only, were pooled, lyophilized and the residue dissolved in a minimal volume of water. Lombricine was further purified by three cycles of crystallization from ethanol. Purity was evaluated by ^{31}P -NMR spectroscopy, TLC, HPLC and direct enzymatic assay using LPK instead of CPK in the assay for creatine (Lowry & Passonneau, 1972).

All biochemicals were from Sigma Chemical Company and Boehringer Mannheim Biochemicals. Glycocyamine was from Fluka Chemical Company (Hauptpage, New York, USA). CP (Boehringer Mannheim) was extremely pure, with virtually no free creatine content. All other chemicals were of reagent grade quality.

Determination of apparent equilibrium constants by conventional enzymatic analysis

Reaction mixtures, consisting of PC, acceptor (arginine, glycocyamine, taurocyamine or lombricine), 2 mmol l^{-1} ATP, 4 mmol l^{-1} magnesium acetate, 50 mmol l^{-1} KCl, 14 mmol l^{-1} 2-mercaptoethanol and 50 mmol l^{-1} Hepes (pH 7.25), were incubated at 35°C in a water bath. For each phosphotransferase reaction, three different combinations of PC and acceptor concentrations (5 mmol l^{-1} PC + 10 mmol l^{-1} X; 10 mmol l^{-1} PC + 5 mmol l^{-1} X; 10 mmol l^{-1} PC + 10 mmol l^{-1} X) were used. At zero time, 25 enzyme units ($\mu\text{mol min}^{-1}$) each of CPK and the appropriate other phosphotransferase (APK, GPK, TPK or LPK) were added to the reaction mixture. After equilibrium had been reached (which occurred in 60 min or less in all cases), 0.67 volumes of cold (-15°C) 12 % (w/v) perchloric acid in 40 % ethanol containing 1 mmol l^{-1} EDTA was injected into each reaction mixture. The resulting solution was centrifuged (4°C , 20 min, $10\,500\text{ g}$). The supernatant was brought to pH 7.5 by titration with 3 mol l^{-1} K_2CO_3 containing 0.3 mol l^{-1} triethanolamine. After sitting on ice for 15 min, the solution was centrifuged (4°C , 20 min, $10\,500\text{ g}$).

Phosphagen (PC, PA, PG, PT or PL) and acceptor (creatine, arginine, glycocyamine, taurocyamine or lombricine) concentrations in the supernatant were assayed immediately by enzyme-linked spectrophotometric assays. Phosphagens were assayed by the assay of Lowry & Passonneau (1972) for PC, using the appropriate phosphotransferase. Acceptors were assayed by the method of Lowry & Passonneau (1972) for creatine, again using the appropriate phosphotransferase. All assays were performed on a Gilford 252-1 UV-VIS spectrophotometer.

Determination of equilibrium constants by ^{31}P -NMR spectroscopy

For ^{31}P -NMR spectroscopic measurement of the time course of attainment of equilibrium, freshly prepared PC and corresponding acceptor stock solutions were enzymatically assayed as above. Equilibrium constant reaction mixtures were then prepared consisting of 10 mmol l^{-1} PC, 10 mmol l^{-1} acceptor, 2 mmol l^{-1} ATP, 4 mmol l^{-1} magnesium acetate, 50 mmol l^{-1} KCl, 14 mmol l^{-1} 2-mercaptoethanol and 50 mmol l^{-1} Hepes at pH 7.25 and transferred to 10 mm (o.d.) NMR tubes with Teflon vortex plugs. After taking enough NMR data for an initial spectrum, 25 enzyme units each of CPK and the appropriate other phosphotransferase were added and additional spectra were generated until equilibrium was attained. Samples were spun and temperature was maintained at $35 \pm 1^{\circ}\text{C}$ using a variable temperature accessory. The pH of some samples was monitored and showed very little change.

NMR spectroscopy was conducted at 61.2713 MHz in a Fourier transform spectrometer. Homogeneity of the magnetic field was optimized by initial shimming on the available water signal. Data were collected in 2 K data sets with a sweep width of $\pm 1000\text{ Hz}$, 90° tip angle and an interval between pulses of 19.5 s. All resonances were proved empirically to be fully relaxed under these experimen-

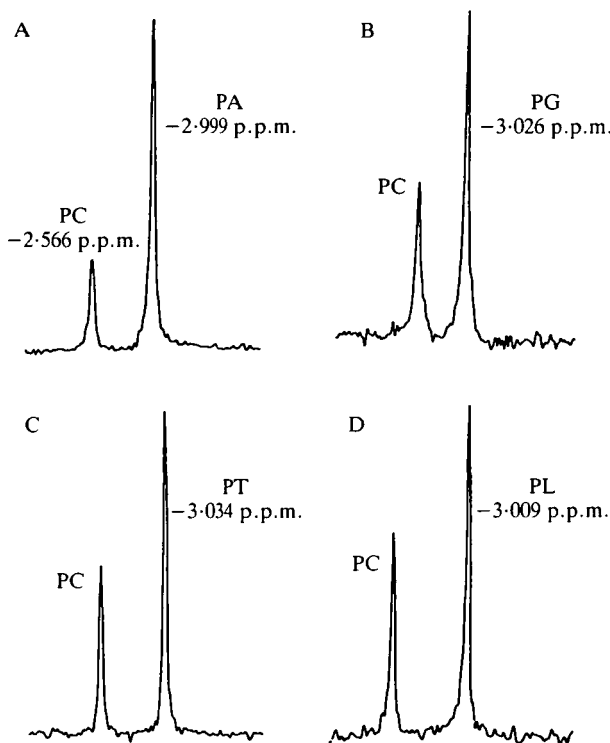


Fig. 2. ^{31}P -NMR spectra of equilibrium mixture of CPK-APK (A), CPK-GPK (B), CPK-TPK (C) and CPK-LPK (D) reactions. Numbers correspond to the chemical shift relative to an external standard of 85% *O*-phosphoric acid. Each spectrum represents the summed data from 72 scans (data acquisitions). Vertical and horizontal scaling vary from spectrum to spectrum.

tal conditions. NMR spectra were generated with a line broadening of 0.1 Hz. Integrals of resonances were estimated using the NTCFTB programs of the Nicolet 1180 computer running the spectrometer. Additional details are listed in the figure legends.

Results

In individual ^{31}P -NMR determinations of K'_{comb} , PA, PG, PT and PL were well resolved from the phosphate resonance of PC (Fig. 2). Furthermore, PA, PG, PT and PL had nearly identical resonance frequencies (Fig. 2), indicating great similarity in the electronic environment of the high-energy phosphate. The average integral ratios PC/PA, PC/PG, PC/PT and PC/PL were 0.360 ± 0.032 , 0.537 ± 0.025 , 0.519 ± 0.024 and 0.583 ± 0.025 , respectively ($\bar{x} \pm 1 \text{ s.d.}$; $N = 4$).

Conventional and ^{31}P -NMR estimates of equilibrium concentrations yielded virtually identical estimates of the K' values for each phosphotransferase reaction (Table 1). The K' values for the APK, GPK, TPK and LPK reactions were all

Table 1. Estimates of the K' values for the APK, GPK, TPK and LPK reactions, relative to K'_{cpk} , measured by conventional and ^{31}P -NMR procedures

Reaction	K' (conventional)* ($N = 6$)	K' (^{31}P -NMR) ($N = 4$)	Overall mean
APK	0.135 ± 0.005	0.129 ± 0.029	0.132
GPK	0.292 ± 0.026	0.288 ± 0.031	0.290
TPK	0.316 ± 0.039	0.270 ± 0.027	0.293
LPK	0.304 ± 0.028	0.341 ± 0.030	0.323

Each value represents a mean \pm 1 s.d.

The overall mean is the average K' for the two methods.

* Conventional and ^{31}P -NMR means for each phosphotransferase are not significantly different.

considerably less than K'_{cpk} . K'_{apk} was the lowest of the four, and the values for GPK, TPK and LPK were roughly 30 % of that of K'_{cpk} .

The validity of the experimental protocol for the determination of the relative K' values for the phosphotransferase reactions was assessed in several ways. First, ^{31}P -NMR spectra of reaction mixtures consisting of PC-arginine or PA-creatine (plus ATP, CPK and APK) showed that both systems approached equilibrium with the same time course and yielded identical equilibrium ratios. Second, experiments were run with mixtures of PA-glycocyamine (plus ATP, GPK and APK) and PA-taurocyamine (plus ATP, TPK and APK). Equilibrium concentrations of reactants were assessed by direct enzymatic assay. The measured equilibrium concentrations yielded K'_{comb} values entirely consistent with K' values determined using the CPK-linked system. The ratios K'_{tpk}/K'_{apk} and K'_{gpk}/K'_{apk} were 1.904 ± 0.095 and 2.243 ± 0.069 , respectively ($\bar{x} \pm 1$ s.d.; $N = 3$).

Discussion

The thermodynamic properties of the phosphagen phosphotransferase and phosphagen hydrolysis reactions

Pronounced thermodynamic differences exist between the invertebrate phosphagen phosphotransferase reactions and CPK (Table 2). Previous studies of APK have shown that K' for APK ranged from 5 to 55 (Morrison, 1973; Rao *et al.* 1976; Graham *et al.* 1986). Assuming a value for K'_{cpk} of 100 (Lawson & Veech, 1979), the present study shows that K'_{apk} is around 13.2 (Table 2). Furthermore, K' values for the GPK, TPK and LPK reactions lie between K'_{apk} and K'_{cpk} , being much closer to K'_{apk} (Table 2). The large discrepancy between the present value for K'_{apk} and the value of 55 determined by Graham *et al.* (1986) can largely be attributed to differences in conditions. Furthermore, changes in pH were not taken into account by Graham *et al.* (1986).

The free energy of hydrolysis of PA is 5.18 kJ mol^{-1} less than that of PC (Table 2). The corresponding ΔG° values for PG, PT and PL are intermediate.

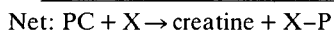
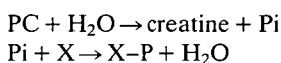
Table 2. *Thermodynamic properties of phosphagen phosphotransferase and hydrolysis reactions*

Reaction (phosphagen)	K'_{xpk}	K' , hydrolysis (mol l^{-1})	ΔG° , hydrolysis (kJ mol^{-1})
CPK	100.0	3.64×10^7	-44.58
APK	13.2	4.81×10^6	-39.40
GPK	29.0	1.06×10^7	-41.42
TPK	29.3	1.07×10^7	-41.45
LPK	32.3	1.18×10^7	-41.70

The apparent equilibrium constants ($K'_{\text{xpk}} = [\text{X}][\text{ATP}]/[\text{X-P}][\text{ADP}]$), apparent equilibrium constants for hydrolysis ($K'_{\text{hydrolysis}}$) and the free energy of hydrolysis of the phosphagen (ΔG°) were estimated using mean K'_{comb} as described below.

Data for CPK are from Lawson & Veech (1979).

The K' value for each phosphotransferase was obtained using the following formula: $K'_{\text{xpk}} = 100/K'_{\text{comb}}$, where 100 is K'_{cpk} , under the current experimental conditions, as estimated using the equations of Lawson & Veech (1979). It must be clearly pointed out that there is still considerable uncertainty as to the *exact* position of K'_{cpk} . Estimates of the free energy of hydrolysis of the various phosphagens are predicted on the following rationale:



K'_{comb} for the net hydrolysis reaction is the same as the net phosphotransferase reaction. Thus, it is possible to use the experimentally determined K'_{comb} to estimate K' values for phosphagen hydrolysis using the following formula: $K'_{\text{hydrolysis}} = 1/K'_{\text{comb}} \times 3.64 \times 10^7 \text{ mol l}^{-1}$, where $3.64 \times 10^7 \text{ mol l}^{-1}$ is K' for CP hydrolysis for Lawson & Veech (1979). The free energy of hydrolysis (ΔG°) is then calculated using the following formula: $\Delta G^\circ = -RT \ln K'$, where K' is the apparent equilibrium constant for hydrolysis for each phosphagen.

Thus, PA, PG, PT and PL exist as a cohort of thermodynamically more stable compounds than PC. This observation is consistent with a prediction made by Ennor & Morrison (1958) based on the resonance properties of the various phosphagens. The chemical stability is probably related to the absence of the methyl group characteristic of PC (Fig. 1). The methyl group in PC eliminates almost all resonance states in the guanidino group, thereby producing an extremely labile phosphorus group (Kalckar, 1941; Oesper, 1950; Pullman & Pullman, 1959; Robin, 1980). The similarities in the chemical shifts of the phosphorus group in NMR spectra of PA, PG, PT and PL (Fig. 2) indicate that the phosphorus moieties have similar electronic environments, which is consistent with similarities in lability. In contrast, the chemical shift of the PC phosphorus is dramatically different from the others. The present observations are not compatible with those of Pin (1965) who found that the heats of hydrolysis of various phosphagens decreased in the following order: $\text{PC} > \text{PA} > \text{PT} > \text{PG}$.

*The importance of K' in terms of the functional roles of phosphagen
phosphotransferase reactions*

As indicated in the Introduction, phosphagens are thought to function as ATP buffers. The importance of the K' values of the corresponding phosphotransferase reactions in this functional role is readily evident from the following expression:

$$\frac{[\text{ATP}]}{[\text{ADP}]} = \frac{[\text{X-P}][\text{H}^+]}{[\text{X}]} K',$$

where X-P = phosphagen and X = acceptor. Thus, at similar H^+ , X-P and X concentrations, the phosphagen phosphotransferase reaction with the highest K' value will be able to buffer ATP at correspondingly higher ATP/ADP ratios. This kind of analysis assumes that the phosphotransferases operate near equilibrium with their substrates *in vivo* and that their respective fluxes are substantially greater than the maximal rates of ATP utilization. This has been clearly established for CPK (Gadian *et al.* 1981; Shoubridge *et al.* 1984) and APK (Newsholme *et al.* 1978; Graham *et al.* 1986). The GPK, TPK and LPK reactions have not been investigated in this regard.

An alternative way to look at these functional differences is by the approach of Kammermeier *et al.* (1982), who constructed curves relating the change in the free energy of hydrolysis of ATP and PC to the degree of hydrolysis of these compounds (Fig. 3). The resulting plots correspond to buffer dissociation curves (Kammermeier *et al.* 1982) and show that when only 5 % of ATP is hydrolysed, the bulk of the PC pool (>60 %) is hydrolysed. Thus, PC can function as an ATP buffer only at relatively high ATP/ADP ratios. If similar hydrolysis curves are constructed for PA, PG, PT and PL, one finds that the buffer dissociation curves are displaced to the left, indicating a capacity for ATP buffering at lower ATP/ADP ratios (Fig. 3). For instance, when 5 % of the ATP pool has been hydrolysed, only about 20 % of the PA pool has been used up. From the point of view of ATP buffering, it is clear that the phosphagens are not all equivalent.

The energetic advantage of PC

PC is the vertebrate phosphagen. However, this compound is also found in protochordates, echinoderms and annelids (Watts, 1975). The distribution of PC and CPK in annelid and echinoderm tissues gives remarkable insight into the energetic advantage of PC over other phosphagen systems. The sperm of polychaetes and echinoderms are flagellated and highly motile. Nearly all polychaete sperm contain CPK *only*, whereas muscles and eggs of the same species may contain other phosphotransferases (van Thoai & Robin, 1969). For instance, the muscles of the polychaetes *Terebella lapdaria*, *Nephtys hombergii*, *Ophelia bicornis* and *Arenicola marina* contain PA, PG, PL and PT, respectively, whereas the sperm contain PC *only* (van Thoai & Robin, 1969). Echinoderm sperm contain CPK *only*, whereas eggs contain APK *only* and muscles contain APK, CPK or even both (Morrison *et al.* 1967). Sperm from arthropods, which are generally not as

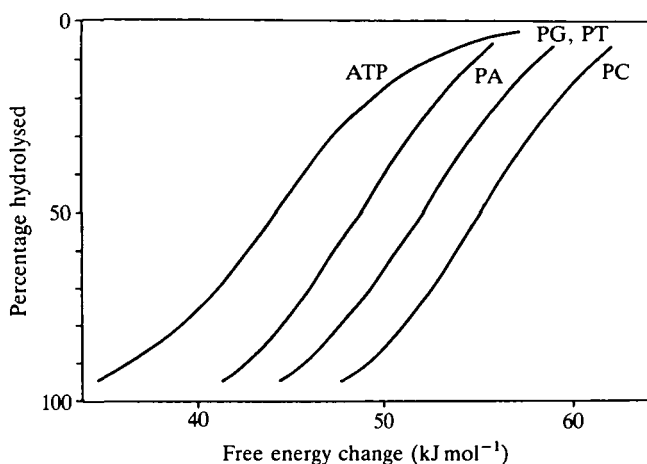


Fig. 3. The free energy change of hydrolysis ($dG/d\xi$) of ATP, PA, PG, PT and PC vs the degree of hydrolysis of the high-energy phosphate. The plot was generated using the following formulae of Kammermeier *et al.* (1982):

$$\frac{dG}{d\xi} = -\Delta G^\circ + RT \ln \frac{[ATP]}{[ADP][Pi]},$$

$$\frac{dG}{d\xi} = -\Delta G^\circ + RT \ln \frac{[X-P]}{[X][Pi]}.$$

The ΔG° value for ATP hydrolysis was from Lawson & Veech (1979) and the ΔG° values for phosphagen hydrolyses are from Table 2. Initial concentrations of reactants, from Kammermeier *et al.* (1982), are as follows: $[ATP] = 8 \text{ mmol l}^{-1}$, $[ADP] = 0.02 \text{ mmol l}^{-1}$, $[Pi] = 1 \text{ mmol l}^{-1}$, $[X-P] = 14 \text{ mmol l}^{-1}$, $[X] = 7 \text{ mmol l}^{-1}$. Curves were then generated by decreasing the high-energy phosphate concentrations taking into account the increase in the concentrations of hydrolysis products. The curve for PL is not shown but would be displaced slightly to the right of the PG/PT curves.

motile as polychaete and echinoderm sperm, contain PA only (Roche *et al.* 1957; Robin, 1980; Robitaille *et al.* 1986).

The presence of CPK in sperm thus appears to be correlated with the existence of a high degree of flagellar activity. The maintenance of a high ATP/ADP ratio in the immediate vicinity of the flagellar proteins is probably of critical importance. Okuno & Brokow (1979) have shown that ADP is an inhibitor of flagellar movement in demembranated sea urchin sperm. Furthermore, Johnson (1985) has recently suggested that the dissociation of ADP and Pi from microtubule-dynein-ATPase is the rate-limiting step during steady-state flagellar activity. Maintenance of low ADP concentrations by the CP/CPK system would tend to promote dissociation of ADP from dynein, thereby enhancing the rate of cycling in the system. Shapiro and coworkers (Tombes & Shapiro, 1985; Tombes *et al.* 1987) have recently provided convincing evidence for a PC shuttle system in sea urchin sperm. The clear-cut conclusion here is that, where there are a number of

phosphagens present in an organism (annelids, some echinoderms), spermatozoa consistently use the CP/CPK system. It should be pointed out that in the 1950s Roche *et al.* (1957) speculated, without elaboration, that the presence of PC in sperm was related to possible higher lability of PC relative to the other phosphagens. Watts (1968) later refuted this for several reasons. My results and analysis are totally compatible with the early speculations of Roche *et al.* (1957).

Excluding certain specialized insect muscles, vertebrate skeletal muscles tend to display higher contractile velocities and more rapid relaxation times than their invertebrate counterparts, which is correlated with differences in actomyosin-ATPase and Ca^{2+} -ATPase activities (Prosser, 1973). The rate of dissociation of ADP from the ADP-actomyosin complex is low, indicating that this is the step that limits the shortening velocity in muscle (Siemankowski & White, 1984). Ventura-Clapier *et al.* (1987) have suggested that the role of PC/CPK is to minimize ADP and maximize ATP concentrations, which then promotes the rapid dissociation of ADP from actomyosin. Presumably, this increases the rate of cross-bridge cycling and enhances the efficiency of the actomyosin-ATPase (Ventura-Clapier *et al.* 1987). In terms of relaxation velocity, Dawson *et al.* (1980) have shown in a vertebrate skeletal muscle that the reciprocal of the rate constant for relaxation is directly proportional to the cytosolic phosphorylation potential ($\Delta G_{\text{ATP}} = \Delta G_{\text{ATP}}^{\circ} + RT \ln [\text{ADP}][\text{Pi}]/[\text{ATP}]$; where $\Delta G_{\text{ATP}}^{\circ}$ is the observed free energy of hydrolysis). This phenomenon is probably due to ΔG_{ATP} effects on the efficiency of the sarcolemmal Ca^{2+} -ATPase (Dawson *et al.* 1980; Kammermeier *et al.* 1982). Thus, the maintenance of a high ATP/ADP ratio would promote rapid relaxation. The major point here is that phosphagen systems which keep ADP at extremely low levels (e.g. PC/CPK) would greatly facilitate contractile velocity and relaxation in muscle. This can be construed as a major advantage of PC over other phosphagen systems.

The advantage of phosphagen phosphotransferase reactions with low K' values

Phosphagen phosphotransferase reactions with relatively low K' values may be advantageous under certain physiological circumstances. We have seen that the CPK reaction is not an effective ATP buffer system at lower ATP/ADP ratios (Fig. 3). Furthermore, reductions in pH will promote net hydrolysis of the phosphagen. However, at any given reduction in pH, phosphotransferase reactions with lower K' values will show a smaller degree of net hydrolysis of phosphagen (see equation in second section of Discussion). Thus, the invertebrate phosphagens might be more effective ATP buffers than PC under reduced intracellular pH conditions. In addition, these phosphagens will be capable of buffering ATP over a much lower range of ATP/ADP ratios.

Insight into the above phenomenon has been provided by the elegant work of Walker and colleagues. Vertebrates readily take up cyclocreatine, an artificial creatine analogue (Annesley & Walker, 1978, 1980; Woznicki & Walker, 1980). Cyclocreatine displaces creatine, is phosphorylated by CPK and accumulates as phosphocyclocreatine (PCC). K' for PCC hydrolysis is 4% of that of PC

(Annesley & Walker, 1977), indicating that PCC is thermodynamically more stable than PC. The presence of PCC tends to delay the onset of ischaemia-induced damage in skeletal muscle (Annesley & Walker, 1980), brain (Woznicki & Walker, 1980), heart muscle (Roberts & Walker, 1982; Turner & Walker, 1985) and skin flaps (Cuono *et al.* 1987). Presumably, this effect is due to the unique ATP buffering properties of PCC, although adaptive changes in the tissue metabolic complement, which have been seen with another creatine analogue (Shields *et al.* 1975; Shoubbridge *et al.* 1985), cannot be excluded.

Returning to invertebrate phosphagens, it is clear that these compounds, particularly PA, would be useful during protracted periods where ATP buffering is required. During environmental hypoxia in many marine invertebrates (reviewed extensively by deZwaan & Putzer, 1985), metabolism must be sustained by anaerobic means for periods of up to several days, although rates of ATP turnover are typically very low under these circumstances. Intracellular acidosis is a common occurrence, especially in molluscs (Ellington, 1983a,b; Walsh *et al.* 1984; Graham & Ellington, 1985), owing to the glycolytic production of protons. A pool of highly labile phosphagen like PC would be rapidly dissipated. In contrast, PA may act as an effective buffer under these circumstances, as there is typically a gradual decline in PA levels during environmental hypoxia (for instance see Ellington, 1983a).

Pluriphosphagens

There is extensive evidence for the existence of more than one phosphagen in tissues of some invertebrates, the so-called pluriphosphagen phenomenon (Robin, 1964). Usually, one phosphagen predominates over the other(s) in terms of concentration (Robin, 1964, 1974). A variety of phosphagen combinations has been seen in the body wall musculature of polychaetes, including PG + PC, PA + PT and PA + PL (van Thoai & Robin, 1969). Furthermore, the polychaete *Travisia forbesi* (van Thoai *et al.* 1964) and several sea urchins (Morrison *et al.* 1967) appear to have *both* PA and PC in roughly similar concentrations in their muscles. Given the pronounced differences in the thermodynamic properties of the APK and CPK reactions, it is difficult to reconcile the coexistence of the two in the same cellular compartment. However, it must be pointed out that the above studies have not directly shown that more than one phosphagen coexists in the same *cell*. Cellular heterogeneity, with different cell populations expressing different phosphagen phosphotransferase activities, might explain the phenomenon of pluriphosphagens. If several phosphagens do exist in the same cell, the phosphotransferase reactions may somehow be functionally compartmentalized within the cell. This possibility, as well as the intracellular localization of various phosphotransferase activities, are currently under investigation in this laboratory. If two phosphokinases are present in the same cellular compartment, one would predict that the corresponding phosphagens would be utilized sequentially during energy deficit.

The evolution of the phosphagen phosphotransferase reactions

Phosphoarginine/arginine phosphokinase appears to represent the most primitive phosphagen system. PG, PT and PL (and presumably PHT and other minor phosphagens) represent intermediate steps towards the acquisition of an extremely labile phosphagen. These compounds constitute dead-end systems, at least in an evolutionary sense (Robin, 1974). The evolution of PC, with the insertion of the methyl group at the *N* portion of the guanidino group, produced an unstable phosphagen resulting in a phosphagen phosphotransferase reaction much more strongly poised, in a thermodynamic sense, in the direction of phosphagen breakdown. The PC/CPK system allows for the maintenance of a higher ATP/ADP ratio which may greatly facilitate the efficient functioning of a variety of cellular motility and transport processes. The question as to whether PG, PT and PL have unique, special roles in the physiology of worm muscle remains to be investigated.

Finally, it should be pointed out that the present studies of the APK, TPK, GPK and LPK reactions were conducted under conditions (especially temperature and ionic strength) far from physiological. K'_{cpk} is highly dependent on pH, ionic strength, temperature and free magnesium concentrations (Alberty, 1969; Phillips *et al.* 1969; Lawson & Veech, 1979). This sensitivity is undoubtedly characteristic of the other phosphagen phosphotransferase reactions. Yancey *et al.* (1982) have shown that the intracellular aqueous environment, from the standpoint of low molecular weight solutes, differs substantially from species to species. Given these differences in the intracellular environment, the diverse array of phosphagen phosphotransferase reactions may display somewhat different properties under prevailing physiological conditions. Thus, fully to appreciate and compare these phosphotransferase reactions, the phosphagens must be evaluated in terms of the respective physiological context under which these reactions operate. It is unclear whether these effects would produce differences significant enough to influence the overall conclusions of this study. A detailed analysis of these possibilities is under way in this laboratory.

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