Biochimica et Biophysica Acta, 971 (1988) 72–84
Elsevier

BBA 12302

Electrophoretic analysis of liver glycogen phosphorylase activation in the freeze-tolerant wood frog

Michael M. Crerar [a], Emina S. David [a] and Kenneth B. Storey [b]

[a] *Department of Biology, York University, North York, and* [b] *Institute of Biochemistry and Department of Biology, Carleton University, Ottawa, Ontario (Canada)*

(Received 26 August 1987)
(Revised manuscript received 19 April 1988)

Key words: Cryoprotection; Glycogen phosphorylase activation; Freeze tolerance; Electrophoretic analysis; (*R. sylvatica* liver)

As an adaptation for overwinter survival, the wood frog, *Rana sylvatica* is able to tolerate the freezing of extracellular body fluids. Tolerance is made possible by the production of very high amounts of glucose in liver which is then sent to other organs where it acts as a cryoprotectant. Cryoprotectant synthesis is under the control of glycogen phosphorylase which in turn is activated in response to ice formation. To determine the mechanism of phosphorylase activation, a quantitative analysis of phosphorylase protein concentration and enzymatic activity in liver was carried out following separation of the phosphorylated *a* and nonphosphorylated *b* forms of the enzyme on native polyacrylamide gels. The results suggest that in gels, the *b* form is completely inactive, even in the presence of AMP and sodium sulfate, whereas the *a* form is active and stimulated 3-fold by these substances. Further, phosphorylase activation appears to arise solely from conversion of the *b* to *a* form of the enzyme without an increase in phosphorylase concentration or activation of a second isozyme. The quantitative analysis presented here should prove generally useful as a simple and rapid method for examining the physiological and genetic regulation of phosphorylase in animal cells.

Introduction

Unique among vertebrate animals, several species of terrestrially hibernating frogs found in northern climates can tolerate the freezing of extracellular body fluids as an adaptation of winter survival [1,2]. Freeze tolerance is facilitated by the accumulation of high concentrations of low-molecular-weight carbohydrate cryoprotectants, glucose in the wood frog, *Rana sylvatica* and two other species, and glycerol in a fourth species [3]. Levels of glucose average about 200 μmol/g wet weight in core organs and blood of *R. sylvatica* (control values 1–5 μmol/g), although amounts as high as 550 μmol/ml (9.9 g%) in blood have been measured [2,4–6]. The production of cryoprotectant relies solely upon the catabolism of liver glycogen reserves and is activated only in response to ice nucleation within the body. Less than 5 min after the initiation of freezing, glucose concentrations in liver and blood are elevated by 6- and 3-fold, respectively, [7] with maximal organ contents reached within 18 h of freezing for liver and 24–48 h for other organs [8].

Abbreviation: CRM, cross-reacting material

Correspondence: M.M. Crerar, Department of Biology, York University, North York, Ontario, M3J 1P3, Canada.

The key to rapid cryoprotectant synthesis is the activation of liver glycogen phosphorylase. Measurements of the activity of this enzyme in the direction of glycogenolysis indicate that phosphorylase activity in crude liver extracts increases from 7- to 13-fold in response to freezing exposure [6,9]. This occurs over the first 1–3 h following extracellular ice nucleation. In addition to this increase in phosphorylase activity, there is a change in the ability of AMP to stimulate enzyme activity; approx. 3-fold stimulation by 1 mM AMP occurs prior to freezing, whereas less than 10% stimulation occurs following freezing [7]. In contrast to the kinetics of phosphorylase activation, this change in AMP sensitivity of the enzyme occurs within minutes after extracellular ice nucleation [7,9]. In comparison to mammalian systems, it is possible that this change in AMP sensitivity might reflect a conversion from the *b* (non-phosphorylated) to the *a* (phosphorylated) form of the enzyme [10]. If this is the case, the slower increase in total phosphorylase activity might reflect either an increase in the actual concentration of the enzyme in liver or activation of a separate isoform of phosphorylase whose activity is not detectable in unfrozen frogs. Alternatively, it is possible that the total increase in phosphorylase activity arises solely from conversion of the *b* to *a* form of the enzyme. In this case, the faster change in AMP sensitivity of the enzyme could not simply be due to the *b* to *a* conversion, but would have to arise from some additional mechanism.

In an attempt to distinguish between these possibilities, we have carried out a quantitative, electrophoretic analysis of phosphorylase activation in *R. sylvatica* liver. Native polyacrylamide gel electrophoresis was employed to separate the *a* and *b* forms of phosphorylase. Following electrophoresis, the phosphorylase activity and enzyme concentration was assessed in control (3° C unfrozen) and freezing exposed (7 d at −3.5° C) frogs by an in situ stain for enzyme activity determined in the direction of glycogen formation and by Western blot analysis [11], respectively. A antibody against bovine liver phosphorylase was used as the probe for Western blot analysis. The results indicate that the *b* form of the enzyme is inactive, even when assayed in the presence of AMP and sodium sulfate, a condition which stimulates the *b* form of the mammalian enzyme when assayed under similar conditions [9,12]. The results also indicate that enzyme activation during freezing is largely the result of conversion from the *b* to *a* form of the enzyme without an increase in phosphorylase concentration or activation of a second isozyme. The electrophoretic analysis presented here provides a general and rapid method for examining the physiological and genetic regulation of phosphorylase expression in animal cells.

Materials and Methods

Animals. R. sylvatica were collected from woodland sites in the Ottawa (Canada) area during early September. Adults (mean weight 8.5 ± 1.2 g) of both sexes were used. Animals were placed in covered plastic boxes containing damp sphagnum moss and were held at a constant 3° C without feeding for 2 weeks before use. One container of frogs was then transferred to an incubator at −3.5° C and animals were frozen; for this size of frog, when frozen with insulating moss, maximal ice formation requires 24–36 h. After 7 d exposure to −3.5° C, frozen animals were removed individually, double-pithed and the liver was immediately dissected out and frozen in liquid nitrogen. Control frogs were held at a constant 3° C before dissection of the liver and freezing in liquid nitrogen. Frozen livers were transferred to −80° C for long-term storage.

Male Sprague-Dawley rats, 3–6 months in age (350–500 g in weight) were obtained from Charles River Inc. (St. Constant. Quebec, Canada). Rats were fed on Purina Rodent Lab. Chow, No. 5001 which was removed 24 h prior to decapitation to lower glycogen stores in tissues for the purpose of minimizing phosphorylase streaking on native polyacrylamide gels.

Preparation of tissue extracts and determination of phosphorylase-specific activity. Liver tissue (approx. 0.05 g) was homogenized in a glass-teflon homogenizer in 0.3 ml of solution containing 50 mM imidazole, (pH 7), 15 mM β-mercaptoethanol, 100 mM sodium fluoride and 1 mM phenylmethylsulfonyl fluoride. In some cases, approx. 0.2 ml of the homogenate was centrifuged through a 1 ml Sephadex G-25 (Pharmacia) column in the above extraction buffer, at 500 rpm for 1 min, in an

attempt to remove small effector molecules. This technique yields a good separation between proteins and low-molecular-weight substances as determined by measuring the fraction of freshly added [γ-^{32}P]ATP (less than 10%) that remained in the protein fraction (more than 80% of the starting protein) following centrifugation. Extracts were assayed for phosphorylase activity in the direction of glycogen formation as described previously [13]. Assays were carried out at 30 °C for 5 min in the absence or presence of 1 mM AMP and 0.5 M sodium sulfate using 10 mM [^{14}C]glucose 1-phosphate, 80 mCi/mol (New England Nuclear) as substrate. 130 mM sodium fluoride was included in the reaction mixture to inhibit phosphorylase phosphatase activity [14]. Following incubation, the reaction mixture was applied to a square of Whatmann 3MM filter paper which was then washed to remove unincorporated substrate and analyzed for radioactivity as described previously [13]. 1 unit of phosphorylase activity is the amount of enzyme required to convert 1 μmol of glucose 1-phosphate into glycogen per min. Protein was determined as described by Bradford [15].

Phosphorylation of glycogen phosphorylase in tissue extracts. Crude liver extracts from control frogs were incubated for approx. 3 h at room temperature in 10 mM $MgCl_2$ and 2 mM ATP in extraction buffer (see above). 6 mM cAMP, although added in some instances, was not necessary for phosphorylation to occur. To determine the position of phosphorylated protein on native polyacrylamide gels, 5 μCi of [γ-^{32}P]ATP, (2000 Ci/mmol, Amersham) was added to 100 μl of the above reaction prior to incubation and 10–20 μl of this was applied to the gel.

Native polyacrylamide gel electrophoresis. Native polyacrylamide gel electrophoresis was carried out as described previously [13]. To detect phosphorylase activity in the gel, gels were washed and incubated in 40 mM glucose 1-phosphate, with or without 3 mM AMP and 0.75 M sodium sulphate as described previously [16]. Gels were incubated for approx. 24 h at 30 °C. Glycogen (0.005%) was added to the gel before polymerization as a primer for glycogen synthesis. Following incubation, glycogen formation was then visualized by treatment of the gel with Gram's iodine stain as described previously [17].

Quantitation of phosphorylase activity in native polyacrylamide gels. Relative quantitative estimates of phosphorylase activity in gels were obtained by comparing the intensity of bands formed in the gel following treatment with Gram's iodine stain (see above). This was accomplished by scanning lanes at a wavelength of 550 nm using an RFT scanning densitometer, type 2950 (Transidyne General Corp.). Areas under the peaks were computed using an electronic graphics calculator (Numonics Corp.). Only those values within the linear range with respect to the amount of protein applied were used for determination of relative phosphorylase activities. As differences in the degree of activity stain were noted between gels, activity estimates were normalized to internal standards containing known amounts of phosphorylase activity when more than one gel was used for analysis in order to minimize differences in incubation conditions and staining between gels.

Quantitation of phosphorylase protein amounts in native polyacrylamide gels. The relative amounts of phosphorylase protein in native polyacrylamide gels was determined by Western blot analysis [11]. Following electrophoresis, protein within the gel was transferred to a nitrocellulose filter (Schleicher and Schuell Inc. BA 85) as described previously [13]. The optimum time for transfer was 4 h at 100 mAmp. Following transfer, the nitrocellulose filter was then processed as described previously [13] except that 3% Blotto (skimmed milk powder, Carnation) was used instead of bovine serum albumin as the blocking reagent to minimize nonspecific antibody binding [18]. A 20-fold dilution of rabbit antiserum generated against bovine liver phosphorylase was used as probe for the analysis. This antiserum was obtained by injecting 10-week-old New Zealand white rabbits (approx. 2.5 kg in weight) first with 0.4 mg and then with three injections (0.3 mg each/month) of purified bovine liver phosphorylase, a kind gift of P.K. Hwang, M. Stern and R.J. Fletterick [19]. Following treatment with antibody, the filter was then treated with 20 μCi ^{125}I-labelled protein A (350–400 Ci/mmol, New England Nuclear) as described previously [13]. The amount of cross-reacting material to phosphorylase antibody was determined by measuring the amount of radioactivity by liquid scintillation analysis in sections of the filter that con-

tained cross-reacting material as determined by autoradiographic analysis using Kodak XAR-5 film. Background levels of radioactivity were subtracted from each value according to the area of the filter analyzed as determined with an electronic graphics calculator (Numonics corp.). Only cross-reacting material values obtained within the linear range of the assay with respect to protein concentration were used for determining phosphorylase amounts. Values were normalized to internal standards when more than one gel was used for analysis. In some instances, filters were treated with alkaline phosphatase conjugated anti-rabbit antiserum (Bio/can Sci. Inc.) as described in the manual entitled Protoblot Immunoscreening System (1986) by Promega Biotec, instead of protein A as the secondary probe. This allowed a more rapid visualization of cross-reacting material in the absence of the quantitative analysis.

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out as described previously [20]. Slab gels (17 × 14 cm, 1.5-mm thick) containing 10% acrylamide in the separating gel were run for 7 h at 50 mAmp. Protein was visualized by staining with 0.25% (w/v) Coomassie brilliant blue R (Sigma) in 50% methanol plus 10% acetic acid. The relative intensities of protein bands within the gel were determined by scanning the gel with a scanning densitometer as described above. The position of liver phosphorylase cross-reacting material in the gel was determined by Western blot analysis as described above except that the time of transfer from the gel to the filter was longer, 12 h at 100 mAmp.

All quantitative values given in the text are presented as the mean plus or minus the standard deviation where n is equal to the number of animals used in the analysis.

Results

Electrophoretic and immunological properties of frog liver phosphorylase

The electrophoretic properties and activity requirements of liver phosphorylase from *R. sylvatica* were examined by subjecting crude liver extracts from nonfrozen (control) and freezing-exposed frogs to native polyacrylamide gel electrophoresis. The position of phosphorylase activity in the gel was determined by an in situ stain for phosphorylase activity (see Materials and Methods). The electrophoretic properties of active frog liver phosphorylase are presented in Fig. 1A in relation to rat liver, muscle and brain phosphorylases. In this experiment, phosphorylase activity was determined in the presence of AMP and high concentrations of sodium sulfate in an attempt to activate the *b* form of the frog liver enzyme. Under these conditions, the rat liver *b* form becomes strongly activated [10,13]. As can be seen in Fig. 1A, liver phosphorylase activity from both control and freezing-exposed frogs migrated as a single band with a mobility similar to that of the *a* form of rat liver phosphorylase [13]. As observed previously [13], rat liver phosphorylase activity appeared streaked in these gels and this is due to the presence of closely migrating *a* and *b* forms of the enzyme that are both active under the incubation conditions used here [13]. Other active liver phosphorylase species from control and freezing-exposed frogs could not be demonstrated in the position of the rat liver *b* form or elsewhere in the gel, even when activity was determined in AMP alone, in sodium sulfate alone or in the absence of both AMP and sodium sulfate.

To determine whether other species of frog phosphorylase were present but inactive in the gel, a Western blot analysis [11] was carried out using as a probe for phosphorylase protein, a polyclonal antibody generated against bovine liver phosphorylase (see Materials and Methods). The position of frog liver cross-reacting material (CRM) on a representative Western blot can be visualized in Fig. 1B. As expected, a band of CRM coincides with the position of the active phosphorylase species present in both control and freezing-exposed frogs. In addition, a second band of CRM is present in control but not in freezing-exposed frogs and this species exhibited a mobility slightly greater than that of the active species. This inactive species exhibited a migration rate similar to that of the rat liver *b* form. No other phosphorylase species from the livers of control and freezing-exposed frogs were observed elsewhere on the gel, even when antibodies generated against rat muscle and brain phosphorylase isozymes were

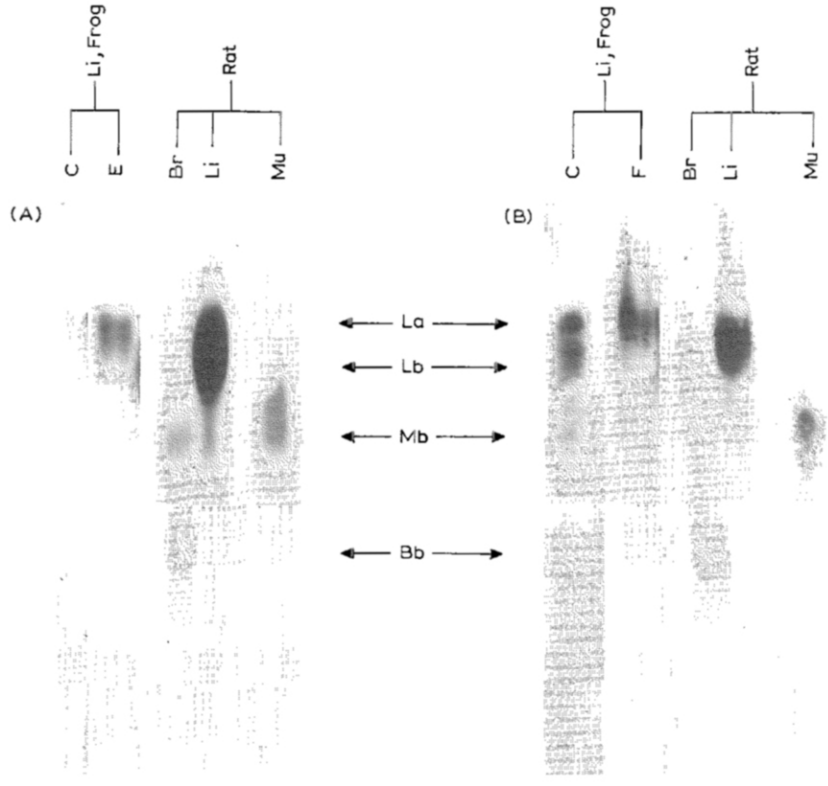

Fig. 1. A comparison of the electrophoretic properties of liver phosphorylase from control and freezing-exposed frogs with those of mammalian phosphorylases on native gels. (A) Phosphorylase activity on native polyacrylamide gels. Crude liver extracts from control (C, 50 μg protein) and freezing-exposed frogs (F, 50 μg protein) as well as extracts from rat tissues; brain (Br, 850 μg protein), liver (Li, 1000 μg protein) and skeletal thigh muscle (Mu, 50 μg protein) were subjected to native polyacryalmide gel electrophoresis. Following electrophoresis, gels were processed for phosphorylase activity in 3 mM AMP and 0.75 M sodium sulfate as described in Materials and Methods. The positions of various isozymic forms of rat phosphorylase as determined by this analysis and previously [13] are indicated down the side of the figure; liver phosphorylase *a* as La; liver phosphorylase *b* as Lb; muscle phosphorylase *b* as Mb and brain phosphorylase *b* as Bb. (B) Western blot analysis of phosphorylase enzymes in (A). Following electrophoresis, protein was transferred from the gel onto a nitrocellulose filter and processed first with bovine liver phosphorylase antibody and then with either ^{125}I-labelled protein A (for C and F) or with alkaline phosphatase conjugated anti-rabbit antiserum (for rat Br, Li and Mu) as described in Materials and Methods. Corresponding photographs of either the autoradiogram or the stained filter are shown here and are presented in relation to the corresponding activity gel in (A). Symbols and amounts of protein applied to the gel are as described in (A).

used as probes. Interestingly, the frog liver phosphorylase species seen here did not cross-react appreciably with polyclonal antibodies generated against rat muscle and brain phosphorylase isozymes. Further, frog muscle phosphorylase exhibited a mobility in gels similar to that of the mammalian muscle form and cross-reacted to a much greater extent with rat muscle phosphorylase antibody than with either mammalian liver or brain phosphorylase antibodies (data not shown). This similarity in the electrophoretic and immunological properties of frog and mammalian isozymes indicates that the phosphorylase isozyme structure has been highly conserved between amphibians and mammals.

Identification of the electrophoretic species of phosphorylase from control and freezing-exposed frogs

To identify the two electrophoretic species of phosphorylase from control frogs, crude extracts were incubated in $MgCl_2$ and ATP to generate the *a* form of the enzyme (see Materials and Methods).

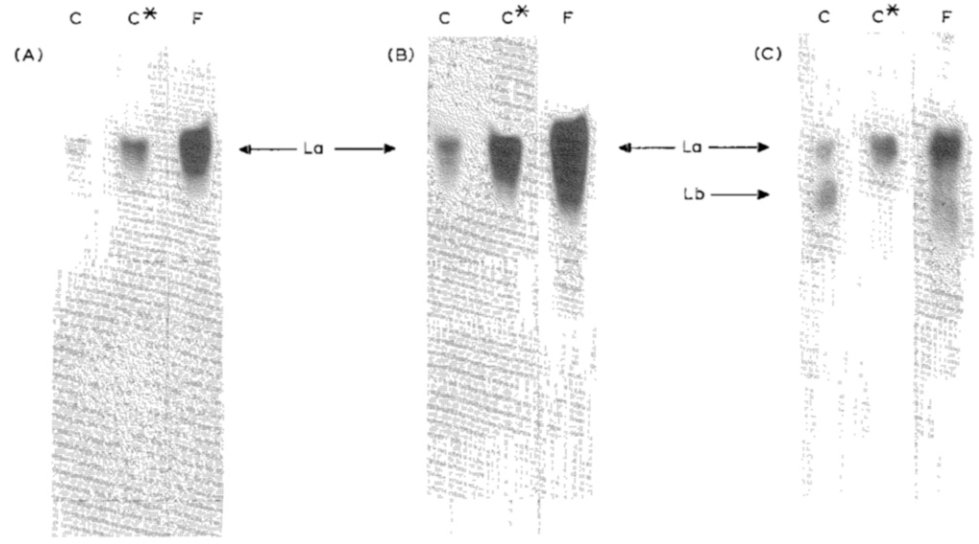

Fig. 2. A comparison of the electrophoretic properties of phosphorylase from control and freezing-exposed livers with that present in in vitro phosphorylated control liver extracts. (A, B) Phosphorylase activity on native polyacrylamide gels assayed in the absence (A) or in the presence (B) of 3 mM AMP and 0.75 M sodium sulfate. Control (C, 15 μg protein), in vitro phosphorylated control (C*, 50 μg protein) and freezing-exposed (F, 100 μg protein) extracts were subjected to native polyacrylamide gel electrophoresis and processed for phosphorylase activity as described in Materials and Methods. The positions of liver phosphorylase *a* (La) and *b* (Lb) are indicated on the side of the figure. (B) Western blot analysis of the phosphorylases in (A). Following electrophoresis, protein was transferred to a nitrocellulose filter and processed first with bovine liver phosphorylase antibody and then with ^{125}I-labelled protein A, as described in Materials and Methods. A representative autoradiogram of the filter is presented here in relation to the activity gels presented in (A) and (B). Symbols and amounts of protein applied to the gel are identical to those presented in (A) and (B).

Crude extracts were then applied to native gels and analyzed either for phosphorylase activity in the absence (Fig. 2A) or presence (Fig. 2B) of AMP and sodium sulfate or for phosphorylase protein using bovine liver phosphorylase antibody as probe (Fig. 2C). As can be seen in Fig. 2, treatment of phosphorylase with $MgCl_2$ and ATP did not change the mobility of the slower migrating, active species. However, following treatment, the faster migrating, inactive species disappeared (Fig. 2C). The most reasonable explanation for the disappearance of the inactive species is that it has shifted in mobility to a position similar to that of the active species. This would then indicate that the active, slower migrating species represents the phosphorylated *a* form of the enzyme and the faster migrating species, the nonphosphorylated *b* form. This effect on the faster migrating species was completely dependent on the presence of ATP and $MgCl_2$ and could not be reproduced with $MgCl_2$ alone. Conversely, in the absence of sodium fluoride, a condition which promotes dephosphorylation of phosphorylase, the active species of phosphorylase disappeared whether or not ATP and $MgCl_2$ were added to the incubation mixture (data not shown).

To obtain further evidence that the active, slow migrating species represents the phosphorylated *a* form of the enzyme, [γ-^{32}P]ATP was included in the incubation mixture during the phosphorylation treatment. Following electrophoresis, the positions of newly phosphorylated proteins in the gel were observed by autoradiographic analysis. The results are presented in Fig. 3A. As can be seen in this figure, there appeared to be only one major protein species in the gel that incorporated radioactive phosphate to a detectable extent and this species migrated with a rate coincident with that of the slower migrating, active species of phosphorylase. This result is consistent with the notion that the slower migrating active species in control frog livers represents the phosphorylated *a*

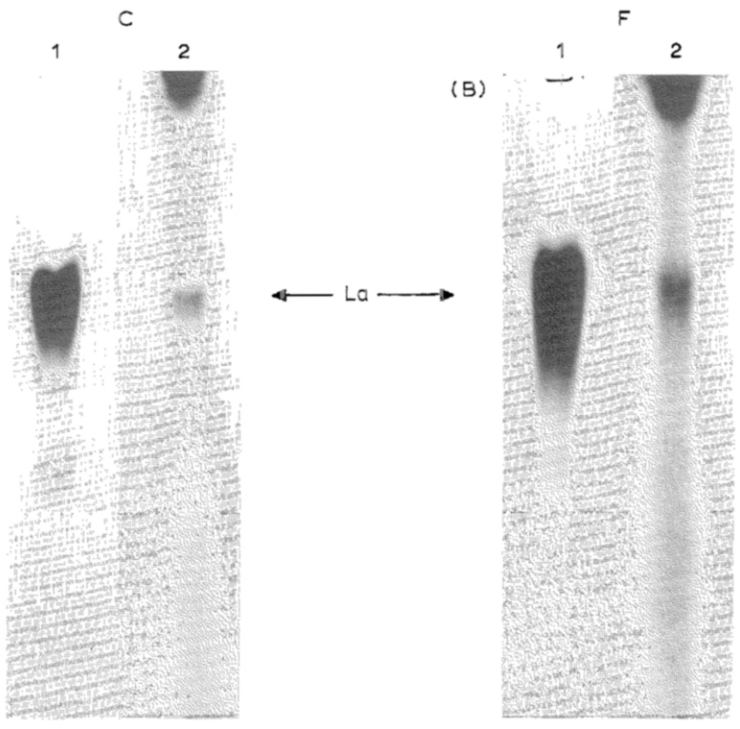

Fig. 3. A comparison of the electrophoretic properties of active frog liver phosphorylase with phosphorylated protein on native polyacrylamide gels. (A) Mobilities of phosphorylase activity and phosphorylated protein from control frogs. A liver extract from a control frog (C) was phosphorylated in vitro in the presence of [γ-^{32}P]ATP as described in Materials and Methods. Following phosphorylation, 50 μg protein/lane was subjected to native polyacrylamide gel electrophoresis. Gels were then stained for phosphorylase activity in the presence of 3 mM AMP and 0.75 M sodium sulfate (1) or subjected to autoradiographic analysis (2) as described in Materials and Methods. La indicates the position of liver phosphorylase *a*. (B) Mobilities of phosphorylase activity and phosphorylated protein from freezing-exposed frogs. Liver extracts from a freezing-exposed (F) frog were processed in a manner similar to that described in (A). Symbols are as described in (A).

form of liver phosphorylase and the faster migrating inactive species represents the nonphosphorylated *b* form of the same enzyme.

Livers from freezing-exposed frogs contained one dominant phosphorylase species that was active and exhibited a mobility similar to that of the phosphorylated species from control livers (see Figs. 1 and 2). ATP-dependent changes in the mobility of this phosphorylase species were not observed. However, incorporation of radioactive phosphate into an electrophoretic species with a mobility similar to that of the active phosphorylase species did occur following treatment of the extract with [γ-^{32}P]ATP and $MgCl_2$ (see Fig. 3B). As a large amount of extract was applied to the gel, it is possible that incorporation was due to the presence in frozen livers of a minor fraction of phosphorylase existing in the *b* state. In accord with this, quantitative estimates of the amount of ^{32}P incorporated into phosphorylase following in vitro phosphorylation (determined by scanning densitometry analysis of autoradiograms of extracts subjected to SDS-polyacrylamide gel electrophoresis) indicated that (3.0 ± 0.7)-fold ($n = 3$ C/F pairs) more radioactivity per unit total soluble protein was incorporated into phosphorylase in control (C) compared to freezing-exposed (F) extracts. Since the dominant phosphorylase species in freezing-exposed frogs is similar in electrophoretic mobility, in turnover number (see below) and in its degree of stimulation by AMP and sodium sulfate (see Fig. 2A and B and below) to that of the slower migrating species in control liver, it is likely that it represents the phosphorylated *a* form of the same enzyme present in control frogs. In accord with this, incubation of the

extract in the absence of sodium fluoride to promote dephosphorylation, yielded an inactive band of CRM on Western blot analysis that migrated with a rate similar to that of the faster migrating *b* form in control extracts (data not shown).

Determination of liver phosphorylase protein and activity levels in control and freezing-exposed frogs

To determine whether it was possible to obtain quantitative estimates of phosphorylase activity and enzyme concentration in gels, varying amounts of crude liver extract from control and freezing-exposed frogs were subjected to native gel electrophoresis. Following electrophoresis, phosphorylase activity was analyzed by scanning the stained gel with a scanning densitometer. Phosphorylase protein concentrations were measured by counting regions of the Western blot that contained radioactivity following treatment with bovine liver phosphorylase antibody and radioactively labelled protein A (see Materials and Methods). Fig. 4A and B represent plots of phosphorylase activity and protein concentration estimates, respectively, determined as a function of the amount of total soluble liver protein applied to the gel. As can be seen in this figure, both activity and protein concentration estimates are linear with respect to the amount of liver extract applied to the gel, up to approx. 60 μg of liver protein in both cases. This indicates that it is possible to quantitate phosphorylase protein and activity levels in frog livers by this analysis as long as values are obtained within the linear portion of these curves.

Quantitative estimates of total phosphorylase protein (*a* plus *b*) and phosphorylase activity levels from gels of liver extracts from control and freezing-exposed frogs are presented in Table I. In this table, values were normalized to total soluble protein present in the liver extracts and are presented as amounts relative to that present in untreated control frogs. As seen in Table I, total (*a* plus *b*) phosphorylase enzyme concentrations (i.e., CRM/mg protein) exhibited little change during freezing of these frogs. The *a* form of the enzyme appeared to be slightly less cross reactive to antibody than the *b* form, (determined by a comparison of values obtained from fresh versus in vitro phosphorylated control liver extracts), and total phosphorylase concentration increased only

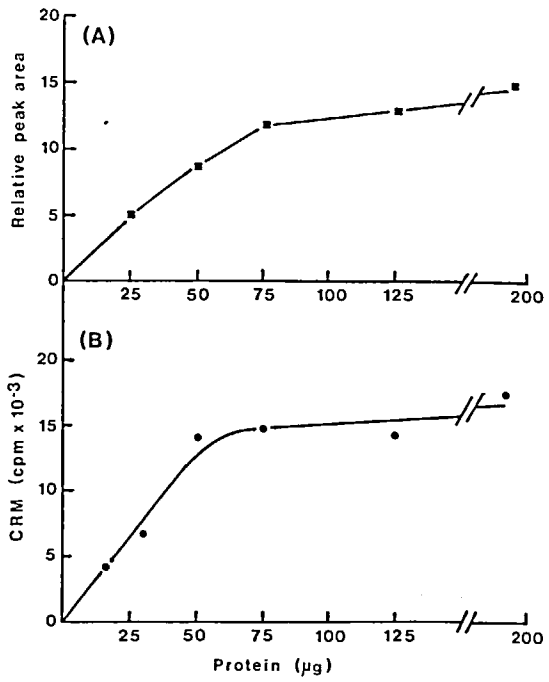

Fig. 4. (A) The relative activity of phosphorylase in gels as a function of the amount of protein applied to the gel. Liver extract from a freezing-exposed frog was subjected to native polyacrylamide gel electrophoresis. The gel was then processed for phosphorylase activity in 3 mM AMP and 0.75 M sodium sulfate and relative activities of phosphorylase were estimated as described in Materials and Methods. (B) The relative amount of cross-reacting material (CRM) to bovine liver phosphorylase antibody in gels as a function of the amount of protein applied to the gel. Liver extract from a freezing-exposed frog was subjected to Western blot analysis and the amount of CRM on the filter was estimated as described in Materials and Methods. Similar results were obtained from control extracts.

slightly, approx. 30%, in the livers of freezing-exposed frogs compared to control frogs when taking this difference in antibody cross-reactivity into account. In contrast, liver phosphorylase activity levels (activity/mg protein) increased approx. 7-fold in livers of freezing-exposed frogs compared to untreated control livers, being slightly higher than the levels observed in in vitro phosphorylated control liver extracts. This indicates that activation of phosphorylase during long-term freezing of *R. sylvatica* occurs mainly by an increase in the inherent turnover number (activity/molecule) of the enzyme with little change in the actual phosphorylase concentration. This increase in phosphorylase turnover number can be explained fully

TABLE I

RELATIVE AMOUNTS OF PHOSPHORYLASE PROTEIN (CRM) AND ACTIVITY IN LIVERS OF CONTROL VERSUS FREEZING-EXPOSED FROGS

All values for untreated control extracts, in vitro phosphorylated control extracts and untreated freezing-exposed liver extracts were determined as described in Materials and Methods. These are presented as averages ± S.D. where n is equal to the number of animals analyzed. For the gel analysis, values are presented relative to those determined in untreated control extracts and activities (act) were determined in the presence of AMP and sodium sulfate. For the analysis in crude cell extracts, activity estimates are presented as munits/mg protein.

Liver tissue	Gel analysis			Analysis in crude extracts (munits/mg protein)	
	act/mg protein	CRM/mg protein	turnover number	minus AMP/sodium sulfate	plus AMP/sodium sulfate
Control (untreated)	1.0±0.8 ($n=4$)	1.0±0.3 ($n=5$)	1.0	42± 26 ($n=6$)	130± 46 ($n=7$)
Control (phosphorylated)	5.7±1.2 ($n=4$)	0.7±0.2 ($n=4$)	5.7	379±245 ($n=8$)	210±147 ($n=8$)
Freezing-exposed (untreated)	6.9±2.5 ($n=5$)	0.9±0.1 ($n=5$)	5.4 [a]	184±110 ($n=7$)	232±106 ($n=7$)

[a] The relative turnover number presented here takes into account the difference in cross reactivity between the *b* and *a* forms and was therefore, calculated as (6.9/0.9·0.7).

by phosphorylation of the enzyme, since control extracts phosphorylated in vitro exhibit a turnover number similar to that demonstrated for phosphorylase in extracts from freezing-exposed frogs (see Table I).

Quantitative estimates of the relative amounts of individual *a* and *b* forms in control frog livers were difficult to obtain, since these forms migrated close together in gels and often overlapped. To further complicate this analysis, the *b* form appeared somewhat streaked (see Figs. 1B and 2C) and this may be due to the presence of both homodimer *bb* and heterodimer *ab* species in this region in accord with the situation in mammals where phosphorylases exist as dimers in vivo [21]. However, rough estimates of these amounts (15–30% *a* form in control frogs and more than 90% *a* form in freezing-exposed frogs) are in agreement with the above-postulated mechanism of activation of phosphorylase during freezing. In summary, these results indicate that during freezing of *R. sylvatica*, phosphorylase is activated mainly by the conversion of a pre-existing inactive, *b* form of the enzyme to an active phosphorylated *a* form with little substantial change occurring in the total phosphorylase (*a* plus *b*) concentration.

For comparative purposes, phosphorylase specific activities were determined in the direction of glycogen formation in crude cell extracts from control and freezing exposed frogs (see Table I). When assayed in the absence of AMP and sodium sulfate, phosphorylase specific activities determined in in vitro phosphorylated control extracts and in extracts from freezing-exposed frogs were 9- and 4.5-fold higher, respectively, than that determined in untreated control extracts. Although a greater degree of variation was noted in crude cell extracts compared to the gel system, these values are in approximate agreement with the relative activities of the extracts on gels.

When assayed in the presence of AMP and sodium sulfate, untreated control extracts exhibited a 3-fold increase in activity compared to the activity determined in the absence of these substances. In comparison to the gel system, the degree of stimulation by AMP and sodium sulfate in control extracts was similar to that observed for the *a* forms in gels from in vitro phosphorylated control extracts ((3.0 ± 1.8)-fold, $n=4$) and freezing-exposed extracts ((3.9 ± 1.0)-fold, $n=3$). The *a* form of the enzyme in untreated control extracts was generally too weak to assess accurately its activity in gels when assayed in the absence of

AMP and sodium sulfate. This might then indicate that stimulation by AMP and sodium sulfate in the control extracts arises from stimulation of the *a* form of the enzyme. However, activities determined in AMP and sodium sulfate in in vitro phosphorylated control and freezing-exposed extracts were not significantly different to those observed in the absence of AMP and sodium sulfate. It is possible that this difference in the degree of sensitivity of the enzyme to AMP and sodium sulfate in the gel system compared to the assay in crude extracts could arise from the presence of interfering enzymes and metabolites in the crude extract that might be removed from the enzyme during electrophoresis. It is unlikely, however, that small effector molecules interfere with enzyme determinations in these crude extracts, since removal of such molecules by Sephadex G-25 chromatography did not give significantly different activity estimates. If the lack of stimulation by AMP and sodium sulfate in crude cell extracts did arise from interfering substances of high molecular weight, then such substances would be expected to interfere only following phosphorylation, since the untreated control extracts exhibit the same degree of stimulation by AMP and sodium sulfate as enzyme activity determined in gels.

SDS-gel analysis of liver proteins in control and freezing-exposed frogs

To determine whether changes in the amounts of major proteins present in liver were occurring during freezing of *R. sylvatica*, crude liver extracts from control and freezing-exposed frogs were subjected to SDS-polyacrylamide gel electrophoresis. Representative gels of liver proteins are presented in Fig. 5A. Visual inspection as well as densitometer tracings of these gels indicated that there were no dramatic changes in the relative amounts of the

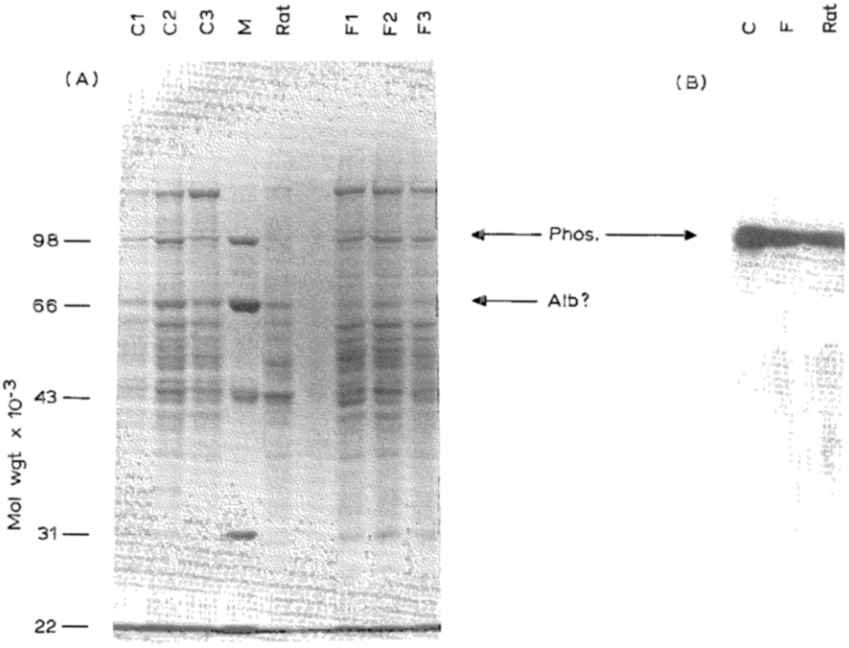

Fig. 5. SDS-gel analysis of liver proteins from control and freezing-exposed frogs. (A) SDS-gel profiles of proteins present in crude cell extracts. Crude liver extracts containing 25 μg protein each from three separate control frogs (C1, C2 and C3), three separate freezing exposed frogs (F1, F2 and F3) and one rat were subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The positions of the molecular weight markers (M), phosphorylase (Phos.) and putative frog liver albumin (Alb?) are indicated on the sides of the figure. (B) Western blot analysis of phosphorylase CRM on SDS gels. Liver extracts (75 μg protein) from control (C) and freezing-exposed (F) frogs and from a rat, were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blot analysis using as probe, bovine liver phosphorylase antibody and ^{125}I-labelled protein A. An autoradiogram of the filter is presented in relation to the corresponding SDS gel in (A).

major liver proteins during freezing, with the exception of one protein that migrated with a mobility similar to that of bovine serum albumin. Attempts to determine whether this protein was frog albumin by Western blot analysis using antibodies generated against bovine serum albumin were unsuccessful due to a lack of cross-reactivity of the antibody. This protein was present in reduced amounts in livers from freezing-exposed compared to control frogs. If this protein does represent frog albumin, observed changes in its amount likely arise as the result of differences in the degree of contamination of the liver preparations with serum rather than as the result of a freezing-stimulated change in the expression of this protein. During liver preparation, attempts were made to remove serum from dissected livers. However, the serum could not be removed completely and it was noted that livers from control frogs did appear to contain more serum than livers from freezing-exposed frogs. This is likely the result of the decreased blood flow that occurs in these animals during freezing [6].

The position of phosphorylase on these gels was determined by Western blot analysis using bovine liver phosphorylase antibody as probe. One major band of CRM was observed that migrated with a mobility similar to that of mammalian phosphorylase (see Fig. 5B). This indicates that frog liver phosphorylase has a subunit molecular weight of approx. 100000. Further, since the migration rates of the frog and mammalian enzymes were similar on native gels, this indicates that both *a* and *b* forms of the frog enzyme likely exist as dimers in vivo similar to the mammalian enzyme [22,23]. Assuming that the major protein species migrating with a mobility similar to that of the CRM on SDS gels is phosphorylase, this would indicate that phosphorylase is present in greater amounts (approx. 5-fold, determined by densitometer scans) in frog livers than in livers of mammals. In accord with this, the specific activity of phosphorylase determined in AMP and sodium sulfate in crude cell extracts from freezing-exposed frogs was 3-fold higher than that determined in rat liver, 67 ± 28 munits/mg protein ($n = 3$). Estimates by densitometer scans of the amount of the species of molecular weight 100000 relative to that of the total protein applied to the gel indicated that phosphorylase comprises $2.8 \pm 0.6\%$ ($n = 5$) and $2.6 \pm 0.2\%$ ($n = 5$) of the total soluble protein present in liver tissue from control and freezing exposed frogs, respectively.

Discussion

Contrary to the situation in cold hardy insects, freeze-tolerant frogs show no anticipatory accumulation of cryoprotectant during cold acclimation [2]. Production is, instead, triggered only when ice formation begins in the body extremities. Regulatory control of glycogen phosphorylase is key to this process providing the means to activate rapidly the enzyme and to maintain this activity in the face of rising and very high levels (200–500 mM) of glucose, the cryoprotectant. Initial studies involving measurements of phosphorylase activity in the presence and absence of AMP suggested the possibility of two mechanisms of phosphorylase activation, the well known *b* to *a* conversion coupled with a major increase in the total (*a* plus *b*) activity of phosphorylase measurable in liver [6,9]. The results of the present study alter this interpretation. The large increase in liver phosphorylase activity apparent in frozen frogs appears to be due largely to the conversion of a completely inactive *b* form of the enzyme into an active *a* form. The phosphorylase *b* from *R. sylvatica* is unusual in its complete lack of activity, even in the presence of both AMP and sodium sulfate, a condition under which the activity of the mammalian liver *b* form is greatly stimulated when assayed in the direction of glycogen formation [10,12]. Interestingly, a phosphorylase *b* that is completely inactive has adaptive significance for the freeze-tolerant frog. Liver accumulates enormous quantities of glycogen (as much as 1000 μmol/g wet weight, in glucose units) during autumn cold hardening [2] and also maintains a high phosphorylase activity and enzyme concentration (see Results), both being key requirements for the rapid synthesis of cryoprotectant in response to freezing (6–8). With both a high substrate concentration and a high enzyme activity, however, strict controls must be in place to regulate glycogenolysis at a low level in the unfrozen state. A phosphorylase *b* which is completely inactive in vivo would serve this purpose. Interest-

ingly, the *b* form of the mammalian liver enzyme shows very little activity relative to the *a* form, even in the presence of AMP and sodium sulfate, when assayed in the physiological direction of glycogenolysis [24]. This indicates that strict control of glycogenolysis in liver by interconversion between inactive *b* and active *a* forms of the enzyme is likely a general phenomenon. An analysis of the structure of frog liver phosphorylase would prove very interesting with respect to the structure/function relationships of allosteric domains within the liver enzyme and within phosphorylases in general [21].

The mechanism that leads to activation of phosphorylase in response to extracellular ice formation in the frog remains a mystery. However, knowing that activation is basically a function of the conversion of the *b* to *a* form of the enzyme will allow future studies to concentrate specifically on the regulation of the enzymes involved in this conversion. Two problems are of immediate interest. The first is the time course of phosphorylase activation. As mentioned in the Introduction, previous studies have indicated that the total rise in phosphorylase activity occurs over a period of 1–3 h. However, a change in the sensitivity of the enzyme to AMP occurs within minutes following extracellular ice formation [6,7,9]. It would be of interest to examine the *b* and *a* forms of the enzyme in our gel system at various times following freezing to determine whether conversion occurs in stages. Related to this, is the second problem of glucose regulation of phosphorylase. During freezing, phosphorylase must somehow remain active under conditions of high glucose concentrations that have been postulated would inactivate the *a* form of the mammalian enzyme and lead to its conversion back to the *b* form [25,26]. We are now in the process of examining the effect of glucose on the activity of the frog liver enzyme in the absence and presence of physiological concentrations of the effector molecules AMP and ATP.

The electrophoretic analysis described here is both rapid and sensitive and allows the examination of not only *a* and *b* interconversions, but also control at the level of protein accumulation during situations where phosphorylase activity is subject to regulation. With this gel system, activity estimates might be expected to be more accurate than in crude cell extracts, since factors that interfere with enzyme activity determinations may be separated from the enzyme following gel electrophoresis. The very close correspondence of the relative turnover numbers of phosphorylase in in vitro phosphorylated control and freezing-exposed extracts in gels attests to the accuracy of the gel analysis (see Table I). It is also possible with this gel system to examine individually the physiological and genetic regulation of multiple isozymes of phosphorylase, even when they are expressed in the same cell type. As demonstrated previously (Ref. 13 and references therein), the *a* and *b* forms of all three isozymes of phosphorylase in mammals; liver, muscle and brain isozymes, can be distinguished by their different migration rates on native gels. Interestingly, differences in the migration rates of the *a* and *b* forms likely reflects not only the charge difference brought about by the presence or absence of the phosphate group, but also surface and or shape differences in the enzyme brought about by the induced allosteric transitions that are promoted by phosphorylation/dephosphorylation [21]. In fact, the relative mobilities of the *a* and *b* forms would be expected to be opposite to what is observed if allosteric conformational changes did not occur. Hence, it should also be possible to examine stable allosteric changes within the *a* and *b* forms of the enzyme by this gel analysis.

Acknowledgements

This work was supported by NSERC No. A8383 and Muscular Dystrophy Association of Canada operating grants to M.M.C. and a NSERC operating grant to K.B.S.

References

1 Schmid, W.D. (1982) Science 215, 697–698.
2 Storey, K.B. (1985) Cryo. Lett. 6, 115–134.
3 Storey, K.B. and Storey, J.M. (1986) Comp. Biochem. Physiol. 83A, 613–617.
4 Storey, K.B. (1987) News Physiol. Sci. 2, 157–160.
5 Storey, K.B. (1987) J. Comp. Physiol. 157, 373–380.
6 Storey, K.B. and Storey, J.M. (1984) J. Comp. Physiol. 155, 29–36.

7 Storey, J.M. and Storey, K.B. (1985) J. Comp. Physiol. 156, 191–195.
8 Storey, K.B. and Storey, J.M. (1986) Can. J. Zool. 64, 49–56.
9 Storey, K.B. and Storey, J.M. (1988) Physiol. Rev. 68, 27–84.
10 Stalmans, W. and Hers, H.-G. (1975) Eur. J. Biochem. 54, 341–350.
11 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
12 Appleman, M.M., Krebs, E.G. and Fischer, E.H. (1966) Biochemistry 5, 2101–2107.
13 David, E.S. and Crerar, M.M. (1986) Biochim. Biophys. Acta 880, 78–90.
14 Cohen, P. (1978) Curr. Top. Regul. 14, 117–196.
15 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
16 Sato, K., Satoh, K., Sato, T., Imai, F. and Morris, H.P. (1976) Cancer Res. 36, 487–495.
17 Takeo. K. and Nakamura, S. (1972) Arch. Biochem. Biophys. 153, 1–7.
18 Johnson, D.A., Gautsch, J.W., Sportsman, J.R. and Elder, J.H. (1984) Gene Anal. Tech. 1, 3–8.
19 Hwang, P., Stern, M. and Fletterick, R.J. (1984) Biochim. Biophys. Acta 791, 252–258.
20 Laemmli, U.K. (1970) Nature (London) 227, 680–685.
21 Fletterick, R.J. and Madsen, N.B. (1980) Annu. Rev. Biochem. 49, 31–61.
22 Stalmans, W. (1976) Curr. Top. Cell. Regul. 11, 51–97.
23 Hers, H.G. (1976) Annu. Rev. Biochem. 45, 167–189.
24 Stalmans, W. and Gevers, G. (1981) Biochem. J. 200, 327–336.
25 Stalmans, W., Laloux, M. and Hers, H.-G. (1974) Eur. J. Biochem. 49, 415–427.
26 Madsen, N.B., Kasvinsky, P.J. and Fletterick, R.J. (1978) J. Biol. Chem. 253, 9097–9101.