The EMBO Journal vol.4 no.13B pp.3735–3742, 1985

Phosphorylation and activation of hamster carbamyl phosphate synthetase II by cAMP-dependent protein kinase. A novel mechanism for regulation of pyrimidine nucleotide biosynthesis

Elizabeth A.Carrey[2], David G.Campbell[1,2] and D.Grahame Hardie[1,2]

[1]MRC Protein Phosphorylation Group, and [2]Biochemistry Department, Dundee University, Dundee DD1 4HN, UK

Communicated by P.Cohen (Dundee)

The trifunctional protein CAD, which contains the first three enzyme activities of pyrimidine nucleotide biosynthesis (carbamyl phosphate synthetase II, aspartate transcarbamylase and dihydro-orotase), is phosphorylated stoichiometrically by cyclic AMP-dependent protein kinase. Phosphorylation activates the ammonia-dependent carbamyl phosphate synthetase activity of the complex by reducing the apparent K_m for ATP. This effect is particularly marked in the presence of the allosteric feedback inhibitor, UTP, when the apparent K_m is reduced by >4-fold. Inhibition by physiological concentrations of UTP is substantially relieved by phosphorylation. Cyclic AMP-dependent protein kinase phosphorylates two serine residues on the protein termed sites 1 and 2, and the primary structures of tryptic peptides containing these sites have been determined:

Site 1: Arg-Leu-Ser(P)-Ser-Phe-Val-Thr-Lys
Site 2: Ile-His-Arg-Ala-Ser(P)-Asp-Pro-Gly-Leu-Pro-Ala-Glu-Glu-Pro-Lys

During the phosphorylation reaction, activation of the carbamyl phosphate synthetase shows a better correlation with occupancy of site 1 rather than site 2. Both phosphorylation and activation can be reversed using purified preparations of the catalytic subunits of protein phosphatases 1- and -2A, and inactivation also correlates better with dephosphorylation of site 1 rather than site 2. We believe this to be the first report that a key enzyme in nucleotide biosynthesis is regulated in a significant manner by reversible covalent modification. The physiological role of this phosphorylation in the stimulation of cell proliferation by growth factors and other mitogens is discussed.

Key words: carbamyl phosphate synthetase II/cAMP-dependent protein kinase/pyrimidine nucleotides/CAD protein

Introduction

In mammals, the first three enzymes on the pathway committed to pyridimine nucleotide biosynthesis (carbamyl phosphate synthetase, aspartate transcarbamylase and dihydro-orotase) are carried on a trifunctional polypeptide which is given the acronym CAD (Jones, 1980; Evans, 1986). The formation of carbamyl phosphate is generally believed to be a rate-limiting step in the pathway, and the synthetase is subject to allosteric regulation, being activated by the nucleotide precursor, phosphoribosylpyrophosphate (PRPP) and inhibited by the end-product, UTP (Tatibana and Shigesada, 1972).

The activities of the CAD complex are clearly essential for proliferation of cells in the absence of exogenous pyrimidines, since inhibitors of these activities block division of cells cultured in minimal medium (Karle and Cysyk, 1984; Aoki *et al.*, 1982; Swyryd *et al.*, 1974). Activation of pyrimidine nucleotide biosynthesis occurs within the first hour of stimulation of quiescent fibroblasts with serum growth factors (Smith and Buchanan, 1979). This occurs in parallel with an increase in uridine uptake (Rozengurt, 1979) and together these events prepare the cell for the rapid synthesis of nucleic acids that occurs later in the G1 and S phases of the cell cycle.

Mitogenic growth factors such as epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1) and platelet-derived growth factor (PDGF, the major growth factor in crude serum) are known to bind to the extracellular domain of specific cell surface receptors, leading to activation of protein (tyrosine) kinase activities which constitute the intracellular domains of the receptors (Heldin and Westermark, 1984). These growth factors also lead to increased phosphorylation of intracellular proteins on serine and threonine residues (Cooper *et al.*, 1982), such as ribosomal protein S6 (Thomas *et al.*, 1982) and the lipogenic enzymes acetyl-CoA carboxylase and ATP-citrate lyase (Holland and Hardie, 1985). Protein phosphorylation would therefore be an attractive mechanism to explain the activation of pyrimidine nucleotide biosynthesis by serum.

Otsuki *et al.* (1981) reported that CAD purified from a rat hepatoma was phosphorylated by cAMP-dependent protein kinase, but failed to detect a significant change in carbamyl phosphate synthetase activity under their assay conditions. We have re-examined this question and find that phosphorylation does produce a marked activation of the enzyme, particularly in the presence of the allosteric activator, UTP. This may represent a key mechanism for activation of pyrimidine nucleotide biosynthesis during stimulation of quiescent cells by growth factors.

Results

Purification and properties of CAD

CAD was purified from an overproducing cell line derived from SV40-transformed Syrian hamster kidney cells (Coleman *et al.*, 1977). The purified preparation contained both carbamyl phosphate synthetase and aspartate transcarbamylase activities in high specific activity [0.3–0.4 μmol/min/mg (using ammonia as substrate) and 8–15 μmol/min/mg, respectively], and exhibited a single major 235-kd polypeptide on SDS-polyacrylamide gel electrophoresis (Figure 1A), corresponding to the trifunctional CAD polypeptide, as reported previously (Coleman *et al.*, 1977). The absorbance index, $A^{1\%}_{280\ nm}$, was 13.0 (see Materials and methods) and the alkali-labile phosphate content was 0.84 ± 0.14 mol/subunit (mean ± standard error of the mean for four preparations).

Phosphorylation of CAD in the absence of protein kinase

When CAD was incubated with 0.1 mM [γ-^{32}P]ATP in the absence of added protein kinase, a negligible amount of phosphate was incorporated into the enzyme (Figure 1B, lane 2). However, if the concentration of ATP was increased to 5 mM, a slow

© IRL Press Limited, Oxford, England

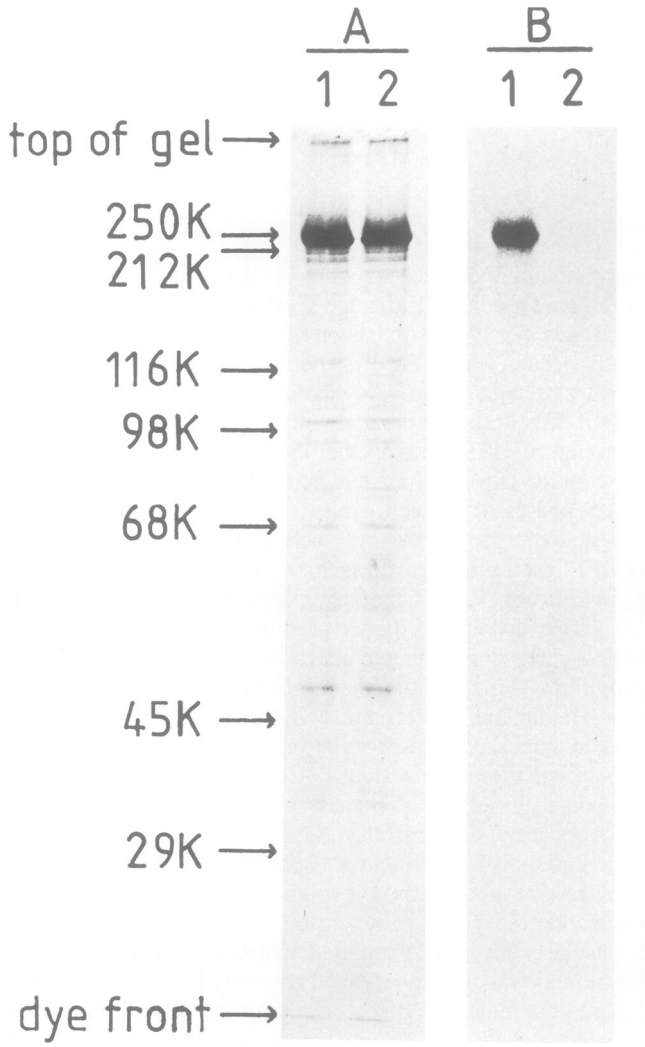

Fig. 1. SDS-polyacrylamide gel analysis of phosphorylated CAD. **Panel A**: Coomassie-blue stained gel; **panel B**: autoradiogram. The protein was incubated with 0.1 mM [γ-^{32}P]ATP and 5 mM $MgCl_2$ either with (**lane 1**) or without (**lane 2**) the catalytic subunit of cyclic AMP-dependent protein kinase and was analysed by electrophoresis as described in Materials and methods. Arrows show the migration of marker proteins in a parallel track (250 K: rabbit mammary fatty acid synthase; 212 K: rabbit muscle myosin heavy chain; 116 K: *E. coli* β-galactosidase; 98 K: rabbit muscle phosphorylase; 68 K: bovine serum albumin; 45 K: hen ovalbumin; 29 K: bovine carbonic anhydrase).

phosphorylation of the enzyme was observed which reached ~0.8 mol/mol after 80 min (Figure 2). Whether this phosphorylation was catalyzed by CAD itself or by a contaminating protein kinase is not clear, but it could be distinguished from the phosphorylation catalyzed by cyclic AMP-dependent protein kinase described below, by several criteria. (i) The extent of phosphorylation by cyclic AMP-dependent protein kinase was not affected when the ATP concentration was reduced from 5 to 0.1 mM. (ii) The endogenous phosphorylation that occurred on incubation of CAD with 5 mM MgATP did not affect the kinetic parameters of the carbamyl phosphate synthetase activity when compared with controls incubated at 0°C without MgATP. (iii) Phosphate was incorporated in the absence of added protein kinase into serine and threonine residues which were recovered after tryptic digestion in peptides distinct from those phosphorylated by cyclic AMP-dependent protein kinase (not illustrated).

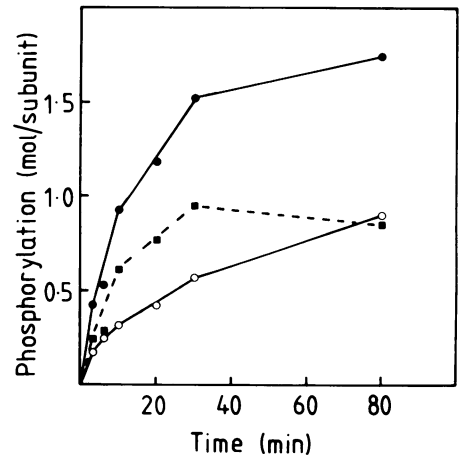

Fig. 2. Time course of phosphorylation of CAD. The protein was incubated with [γ-^{32}P]ATP in the presence (filled circles) or absence (open circles) of the catalytic subunit of cyclic AMP-dependent protein kinase. The broken line (squares) shows the protein kinase-dependent phosphorylation (i.e., phosphorylation in the presence of the kinase minus the phosphorylation in its absence).

In all subsequent experiments involving phosphorylation, control experiments were carried out in which CAD was incubated with MgATP without protein kinase, and data have been corrected for incorporation of phosphate into CAD in these control incubations.

Phosphorylation of CAD in the presence of cyclic AMP-dependent protein kinase

CAD was phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase (Figure 2), and the radioactivity was incorporated exclusively into the 235-kd CAD polypeptide (Figure 1B, lane 1). After correction for endogenous phosphorylation, the stoichiometry of phosphorylation was 0.99 ± 0.07 mol/subunit (mean ± standard error of the mean for six different enzyme preparations).

The initial rate of phosphorylation of CAD by cyclic AMP-dependent protein kinase is compared in Table I with rates of phosphorylation of other established physiological substrates of the protein kinase. At the substrate concentration used (5 μM), the phosphorylation of CAD is much slower than that of glycogen synthase or phosphorylase kinase, but comparable with that of acetyl-CoA carboxylase.

Effect of phosphorylation on ammonia-dependent carbamyl phosphate synthetase activity

CAD was incubated with unlabelled 5 mM ATP with or without the catalytic subunit of cyclic AMP-dependent protein kinase and the carbamyl phosphate synthetase activity was measured at a range of ATP concentrations. In parallel incubations using [γ-^{32}P]ATP, the phosphate incorporation catalyzed by cyclic AMP-dependent protein kinase was found to be 0.96 mol/subunit. Incubations were carried out at high (5 mM) ATP concentrations because this was found to stabilize carbamyl phosphate synthetase activity during incubation at 30°C. The results are shown in Figure 3, and the kinetic parameters determined from these data are summarised in Table II.

The dependence of initial velocity on ATP concentration was distinctly sigmoidal (Hill coefficients >2), and the allosteric effectors UTP and PRPP increased and decreased, respectively, the apparent K_m for ATP, with no significant effect on V_{max}. This is similar to previous reports in which the overall glutamine-dependent activity was measured (Tatibana and Shigesada, 1972).

Table I. Comparison of initial rates of phosphorylation of CAD and other physiological substrates of cyclic AMP-dependent protein kinase

Substrate	Concentration (mg/ml)	Relative rate (%)
CAD	1.18	1.5
Phosphorylase kinase	1.68	100
Glycogen synthase	0.44	61
Histone H1	1.0	46
Acetyl-CoA carboxylase	1.2	2.9

Data are expressed as percentages of the initial rate of phosphorylation of phosphorylase kinase. Substrates were used at the indicated final concentrations, which are equivalent to 5 μM with the exception of histone H1 (50 μM).

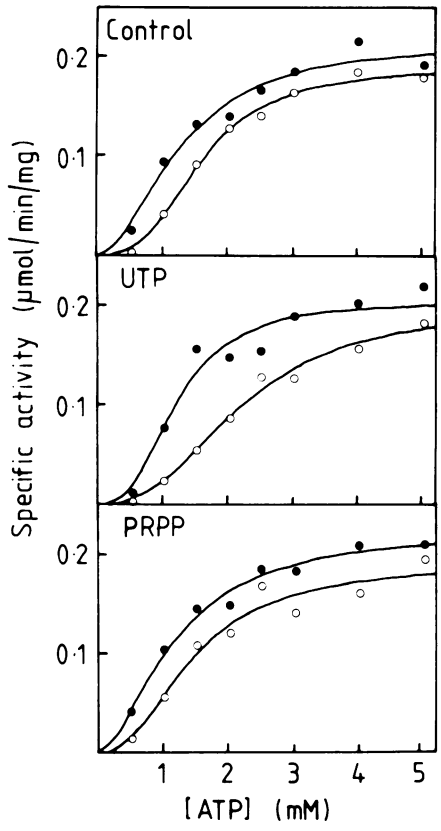

Fig. 3. Dependence of carbamyl phosphate synthetase activity on ATP concentration. CAD was pre-incubated with MgATP in the presence (filled circles) or absence (open circles) of cyclic AMP-dependent protein kinase. Assays were performed in the presence of 2 mM UTP or 0.1 mM PRPP, or in the absence of either effector (control).

For enzyme incubated in the presence of protein kinase, a marked activation was observed which was accounted for by a decrease in K_m for ATP, with no change in V_{max}. This was apparent in the presence or absence of allosteric effectors, but was particularly marked in the presence of the inhibitor, UTP. Figure 4 shows the inhibition by UTP measured at concentrations of ATP and Mg^{2+} (1.5 and 5 mM, respectively) that are suboptimal, but nearer to the physiological values than those used in the standard assay. It can be seen that phosphorylation by cyclic AMP-dependent protein kinase relieves inhibition by the feedback inhibitor, with the concentration of UTP giving 50% inhibition being raised from 0.36 mM to 0.7 mM. At physiological concentrations of UTP ($\sim$0.3 mM) phosphorylation activates the enzyme $\sim$2.5-fold.

Table II. Kinetic parameters of ammonia-dependent carbamyl phosphate synthetase after incubation of CAD with 5 mM MgATP in the presence (phosphorylated) or absence (control) of cyclic AMP-dependent protein kinase

Allosteric effector present	Parameter	Phosphorylated CAD	Control CAD
None	V_{max} (μmol/min/mg)	0.22 ± 0.01	0.19 ± 0.01
	K_m ATP (mM)	1.66 ± 0.34	4.00 ± 0.44
	$S_{0.5}$ ATP (mM)	1.30	1.62
	Hill coefficient	2.0	2.9
UTP (2 mM)	V_{max} (μmol/min/mg)	0.20 ± 0.01	0.20 ± 0.001
	K_m ATP (mM)	1.73 ± 0.52	7.69 ± 1.06
	$S_{0.5}$ ATP (mM)	1.22	2.26
	Hill coefficient	2.7	2.5
PRPP (0.1 mM)	V_{max} (μmol/min/mg)	0.23 ± 0.01	0.19 ± 0.01
	K_m ATP (mM)	1.37 ± 0.22	2.33 ± 0.65
	$S_{0.5}$ ATP (mM)	1.20	1.45
	Hill coefficient	1.7	2.3

Parameters were determined by computer fitting to the Hill equation. Results for V_{max} and apparent K_m for ATP are shown ± standard deviation. $S_{0.5}$ ATP is the concentration of ATP giving half-maximal velocity, which is given by $\sqrt[n]{K_m}$, where n is the Hill coefficient. Further details are given in Materials and methods.

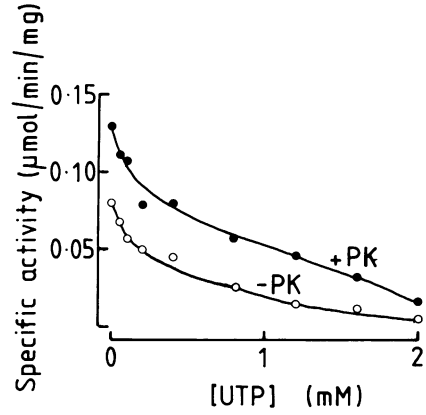

Fig. 4. Effect of UTP on the activity of CAD. CAD was pre-incubated with MgATP in the presence (filled circles) or absence (open circles) of cyclic AMP-dependent protein kinase and assayed at 1.5 mM ATP and 5 mM Mg^{2+} and varying concentrations of UTP.

Analysis of phosphorylation sites

14 mg of CAD was phosphorylated using 0.1 mM [γ-^{32}P]ATP and the catalytic subunit of cyclic AMP-dependent protein kinase, to a stoichiometry of 0.7 mol phosphate/subunit, and was digested with trypsin. Analysis of the digest using reversed phase h.p.l.c. revealed that the bulk of the radioactivity was distributed in two peptides, T1 and T2, which accounted for 52% and 36%, respectively, of the total radioactivity recovered from the column (Figure 5A). These peptides were purified by gel filtration and reversed phase h.p.l.c. as described under Materials and methods. The amino acid compositions and phosphate content of the purified peptides are shown in Table III, and amino acid sequence data obtained from the peptides is summarised in Table IV.

Partial acid hydrolysis of T1 and T2, followed by electrophoresis at pH 1.9, showed that phosphoserine was the only radioactive phosphoamino acid in each case (data not shown). The complete primary structures of T1 and T2 were elucidated by automated liquid-phase Edman degradation. T1 was an eight residue peptide with the sequence Arg-Leu-Ser-Ser-Phe-Val-Thr-

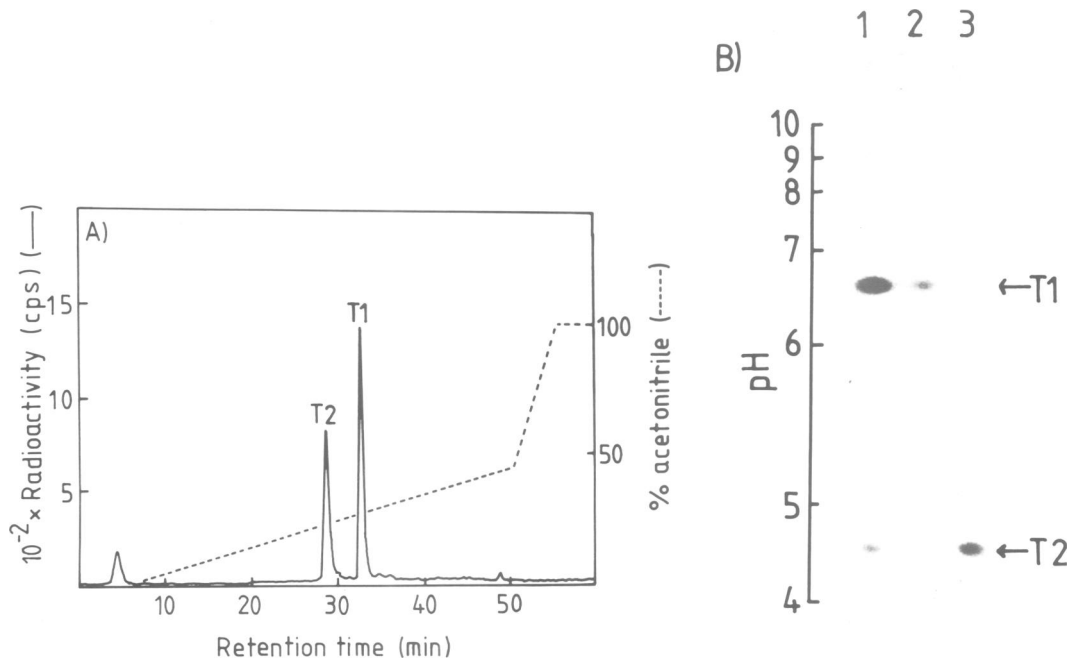

Fig. 5. Separation of tryptic peptides T1 and T2 by reversed phase h.p.l.c. (**A**) and isoelectric focussing (**B**). (**A**) A tryptic digest of CAD phosphorylated using [γ-^{32}P]ATP and the catalytic subunit of cyclic AMP-dependent protein kinase was analysed by reversed phase h.p.l.c. in 0.1% (v/v) trifluoroacetic acid as described in Materials and methods. (**B**) Thin layer isoelectric focussing of (**1**) a crude tryptic digest [as in (**A**)]; (**2**) purified peptide T1; (**3**) purified peptide T2. The photograph shows an autoradiogram of the dried gel. The pH gradient was determined using isoelectric point markers.

Table III. Amino acid compositions and phosphate contents of peptides T1 and T2 purified from 60 nmol of phosphorylated CAD

	T1	T2
Asx	–	0.8(1)
Glx	–	2.0(2)
Ser	1.5(2)	0.7(1)
Gly	0.4(0)	1.1(1)
His	–	0.4(1)
Arg	1.0(1)	1.0(1)
Thr	0.9(1)	–
Ala	–	1.7(2)
Pro	–	2.6(3)
Val	1.1(1)	–
Ile	–	0.9(1)
Leu	1.1(1)	1.2(1)
Phe	0.8(1)	–
Lys	0.9(1)	1.0(1)
Total residues	(8)	(15)
Alkali-labile phosphate	0.76	0.62
^{32}P-phosphate	0.66	0.26
Yield of peptide (nmol)	11	24

The phosphate contents were determined by direct estimation of alkali-labile phosphate and from the ^{32}P radioactivity. Data are expressed as molar ratios with respect to arginine, and impurities with a ratio <0.3 are omitted. Tyr, Met and Cys were absent from both peptides. Values for serine and threonine are not corrected for losses during hydrolysis. Numbers in parenthesis indicate residues determined by sequencing (see Table IV).

Lys. The fact that trypsin did not remove the N-terminal arginine suggested that Ser-3 was the phosphorylated residue, since a phosphoserine two residues on the C-terminal side of an arginine residue has been found previously to prevent tryptic cleavage (Cohen *et al.*, 1975; Proud *et al.*, 1977). This was confirmed by the large burst of radioactivity released after three cycles of degradation (Table IV) although these data could not rule out the possibility that serine-4 was also partially phosphorylated. However, this latter possibility was excluded by two further observations. (i) Thin-layer isoelectric focussing revealed that T1 had a single isoelectric point of ~ 6.5, as expected for the monophosphorylated derivative (Figure 5B). This analytical method would clearly resolve singly and doubly phosphorylated forms of the peptide if both were present (see e.g., Hemmings *et al.*, 1981). (ii) The phosphate content of T1 (Table III) was also consistent with T1 being a monophosphorylated derivative.

T2 was a 15-residue peptide with the sequence Ile-His-Arg-Ala-Ser-Asp-Pro-Gly-Leu-Pro-Ala-Glu-Glu-Pro-Lys. Serine-5 is the only possible phosphorylation site in this peptide, as confirmed by the burst of radioactivity at cycle 5 during automated Edman degradation (Table IV).

The phosphate contents of peptides T1 and T2 were determined by two methods: (i) calculation from the ^{32}P content per mol of peptide, using the known specific radioactivity of the [γ-^{32}P]ATP used for the original phosphorylation; and (ii) direct spectrophotometric determination of alkali-labile phosphate. For T1 these methods were in good agreement: however, for T2 the akali-labile phosphate content was significantly higher than that determined from the ^{32}P-radioactivity (Table III). This indicates that T2 was partially phosphorylated (~ 0.35 mol/subunit) in the original CAD as isolated. This important finding is considered further in the Discussion. In the remainder of the paper the phosphorylation sites contained within peptides T1 and T2 will be referred to as sites 1 and 2, respectively.

Correlation between phosphorylation of site 1 and activation of carbamyl phosphate synthetase

Figure 6 shows the correlation between carbamyl phosphate synthetase activity and the occupancy of sites 1 and 2 measured during a time course of phosphorylation by cyclic AMP-dependent protein kinase. Activation of carbamyl phosphate synthetase showed a better correlation with the phosphorylation of site 1

Table IV. Summary of data on sequencing of peptides T1 and T2 by automated Edman degradation

Cycle no.	T1			T2		
	Residue identified	Yield (nmol)	Radioactivity (c.p.m.)	Residue identified	Yield (nmol)	Radioactivity (c.p.m.)
1	Arg	1.1	105	Ile	3.6	60
2	Leu	1.7	149	His	3.3	83
3	Ser	0.7	2804	Arg	3.6	89
4	Ser	0.9	2187	Ala	2.1	218
5	Phe	1.1	1707	Ser	1.3	3823
6	Val	1.0	1212	Asp	1.5	2480
7	Thr	0.2	910	Pro	0.8	1716
8	Lys	0.4	690	Gly	1.0	1297
9				Leu	1.1	979
10				Pro	1.0	879
11				Ala	1.0	697
12				Glu	0.9	590
13				Glu	1.2	577
14				Pro	0.4	516
15				Lys	0.1	480

Amounts loaded were: 2.0 nmol, 57 000 c.p.m. (T1) and 5.0 nmol, 92 000 c.p.m. (T2). Data shown are the yield of each PTH amino acid recovered by reversed phase h.p.l.c., and the radioactivity in the butyl chloride extract at each cycle. The thiazoline derivative of phosphoserine is unstable and breaks down rapidly during the cleavage reaction to yield inorganic phosphate, which is poorly extracted by butyl chloride. This leads to a 'burst' of radioactivity at the position of the phosphoseryl residue and a trail of radioactivity at subsequent cycles.

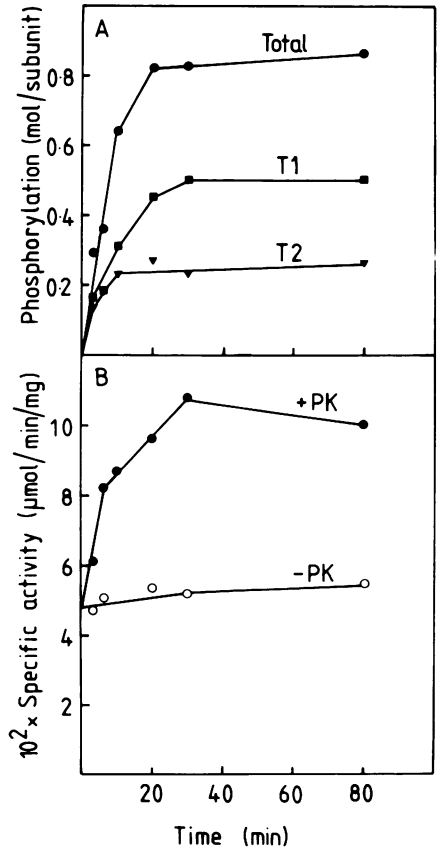

Fig. 6. Occupancy of sites 1 and 2 (**A**) and ammonia-dependent carbamyl phosphate synthetase activity (**B**) during a time course of phosphorylation. Experiments were carried out in parallel using [γ-^{32}P]ATP (**A**) and unlabelled ATP (**B**). (**A**) Total phosphorylation catalyzed by cyclic AMP-dependent protein kinase is denoted by circles, phosphorylation of T1 by squares and T2 by triangles. Distribution of radioactivity between T1 and T2 was determined by densitometry of autoradiograms after thin layer isoelectric focussing as in Figure 5B. (**B**) Carbamyl phosphate synthetase activity of CAD incubated in the presence (filled circles) or absence (open circles) of cyclic AMP-dependent protein kinase. Assays were carried out using 1.5 mM ATP and 2 mM UTP.

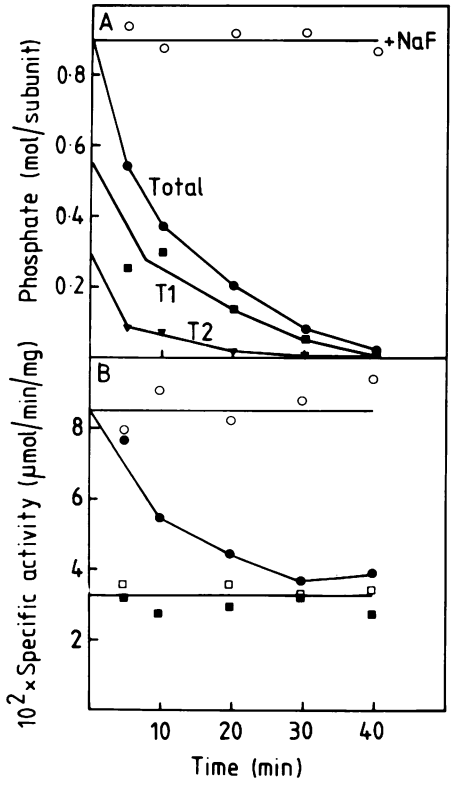

Fig. 7. Occupancy of sites 1 and 2 (**A**) and carbamyl phosphate synthetase activity (**B**) during a time course of dephosphorylation by protein phosphatase-1. The catalytic subunit of protein phosphatase-1 was used at 1300 U/ml in the absence of Mn^{2+} ions. Experiments were carried out in parallel using CAD phosphorylated with [γ-^{32}P]ATP (**A**) or unlabelled ATP (**B**). Occupancy of sites 1 and 2 and enzyme activity were determined as for Figure 6. (**A**) Total protein kinase-catalyzed phosphate content (circles), and occupancy of site 1 (squares) and site 2 (triangles) during treatment with protein phosphatase-1. The total protein kinase-dependent phosphate content during incubation with protein phosphatase-1 in the presence of 50 mM NaF is denoted by open circles. (**B**) Carbamyl phosphate synthetase activity for CAD pre-incubated with MgATP either with (circles) or without (squares) cyclic AMP-dependent protein kinase. The enzyme was incubated with protein phosphatase-1 either with (open symbols) or without (closed symbols) 50 mM NaF.

E.A.Carrey, D.G.Campbell and D.G.Hardie

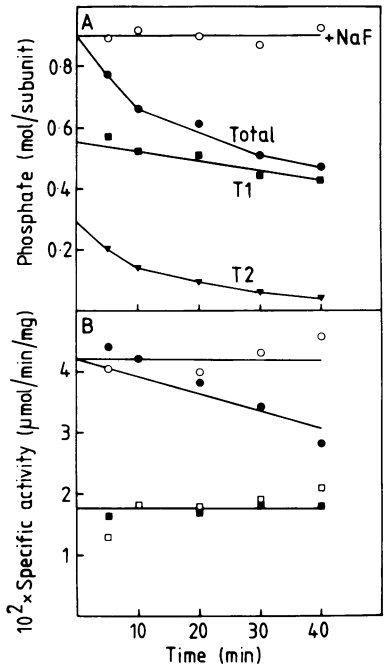

Fig. 8. Occupancy of sites 1 and 2 (**A**) and carbamyl phosphate synthetase activity (**B**) during a time course of dephosphorylation by protein phosphatase-2. The design of the experiment and the symbols used are as in Figure 7 except that the catalytic subunit of protein phosphatase-2A (300 U/ml) replaced protein phosphatase-1, and the carbamyl phosphate synthetase assays were carried out using 5 mM $MgCl_2$.

rather than site 2. Site 2 was initially phosphorylated as rapidly as site 1, but its phosphorylation reached a plateau at ~10 min, whereas at this time the carbamyl phosphate synthetase activity was still rising.

Reversal of phosphorylation of CAD by protein phosphatases and correlation of carbamyl phosphate synthetase activity with dephosphorylation of site 1

^{32}P-Labelled CAD could be dephosphorylated using the catalytic subunits of either protein phosphatase-1 or -2A purified from rabbit skeletal muscle (Figures 7 and 8). In the case of protein phosphatase-1 this was associated with a complete reversal of the effect of phosphorylation on carbamyl phosphate synthetase activity, i.e., a decrease in activity measured at 2 mM UTP to the control value. Analysis by isoelectric focussing of the occupancy of sites 1 and 2 during this dephosphorylation revealed a better correlation between enzyme activity and the dephosphorylation of site 1 rather than site 2 (Figure 7A, B). Reversal of the effect of phosphorylation on carbamyl phosphate synthetase activity by protein phosphatase-2A was only partial, but again correlated better with dephosphorylation of site 1, which in this case was much slower than that of site-2 (Figure 8A, B).

Addition of the phosphatase inhibitor sodium fluoride completely prevented dephosphorylation and inactivation of carbamyl phosphate synthetase by both protein phosphatase-1 and -2A (Figures 7 and 8).

Discussion

Our data strongly suggest that phosphorylation of CAD has an important physiological role, since it is associated with a substantial (~2.5-fold) increase of carbamyl phosphate synthetase activity at physiological concentrations of ATP and UTP. That phosphorylation is the cause of activation is clearly shown by the observation that it can be reversed by treatment with protein phosphatases. Although there was a better correlation between carbamyl phosphate synthetase activity and the occupancy of site 1, rather than site 2, during both phosphorylation and dephosphorylation, the possibility that phosphorylation of the latter site has a minor effect on activity cannot yet be ruled out. Other possible functional effects of site 2 phosphorylation remain to be tested.

Otsuki *et al.* (1981) previously reported that phosphorylation of CAD purified from rat hepatoma produced only a slight increase (~10–20%) in carbamyl phosphate synthetase activity. Their failure to observe the large effects reported in this paper can be attributed to at least two causes: (i) the low stoichiometry of phosphorylation achieved (0.5 mol/subunit); (ii) activity was not measured in the presence of the allosteric inhibitor UTP. An additional difference from our experiments is that Otsuki *et al.* (1981) measured carbamyl phosphate synthetase using glutamine as substrate (i.e., reactions 1 + 2 below), whereas we measured only the second partial reaction, using ammonia as substrate (reaction 2):

$$\text{glutamine} + H_2O \rightarrow \text{glutamic acid} + NH_3 \quad (1)$$

$$NH_3 + 2ATP + HCO_3^- \rightarrow \text{carbamyl phosphate} + 2ADP + Pi \quad (2)$$

We have found that the glutamine-dependent carbamyl phosphate synthetase (reactions 1 + 2) is more thermolabile than the ammonia-dependent activity (reaction 2), suggesting that the glutaminase activity (reaction 1) is catalysed by a distinct site on the enzyme (E.A.Carrey, in preparation), as in the case of carbamyl phosphate synthetase from *Escherichia coli* (Raushel *et al.*, 1978). However, preliminary experiments have shown that phosphorylation activates the glutamine- and ammonia-dependent activities to a similar extent.

Although the rate of phosphorylation of CAD by cyclic AMP-dependent protein kinase is low compared with that of the enzymes of skeletal muscle glycogen metabolism, it is comparable with that of the fatty acid biosynthetic enzyme, acetyl-CoA carboxylase (Table I). There is good evidence that the latter enzyme is phosphorylated in intact cells by cyclic AMP-dependent protein kinase (Holland *et al.*, 1984, 1985). It seems reasonable that while regulation of skeletal muscle glycogen metabolism in response to adrenaline must occur within seconds, slower changes in phosphorylation may be acceptable for regulation of fatty acid and pyrimidine nucleotide biosynthesis.

Many of the physiological substrates for cyclic AMP-dependent protein kinase contain two adjacent basic residues N-terminal to the phosphorylation site, and the importance of this structural feature as a specificity determinant has been established (reviewed by Cohen, 1985). Site 1 of CAD also has this feature since the presence of a lysine or arginine preceding the N-terminal arginine can be inferred from the fact that this is a tryptic peptide. Site 2 contains only a single basic residue two residues N-terminal to the phosphoserine, which may explain its relatively slow rate of phosphorylation.

The observation that the total phosphate content of peptide T2 (0.61 mol/mol) was greater than that calculated from its ^{32}P-radioactivity (0.26 mol/mol) suggests that this site was already partially phosphorylated in the enzyme as isolated. This site would also account for a significant part of the 0.84 mol alkali-labile phosphate per subunit measured in the intact protein. Since no particular precautions were taken to prevent dephosphorylation of CAD during isolation, the phosphorylation of site 2 (and other sites) may have been even greater in the intact cells.

Our results demonstrate that the catalytic subunits of protein phosphatases-1 and -2A will dephosphorylate CAD labelled at both sites 1 and 2, albeit slowly. The dephosphorylation of CAD was very much slower than that of glycogen phosphorylase, a

finding which was partly, but not entirely, accounted for by inhibition of the phosphatase due to the dimethylsulphoxide and glycerol included to stabilize carbamyl phosphate synthetase activity (D.G.Hardie, unpublished). Further work is required to establish whether these or other protein phosphatases are active against CAD *in vivo*.

To our knowledge this paper represents the first report that an enzyme of nucleotide metabolism is regulated by covalent modification in a manner which may have physiological significance. The results are also novel because nearly all other eukaryotic biosynthetic enzymes that are regulated by phosphorylation are inactivated, rather than activated, by this modification. Pyrimidine nucleotide biosynthesis is required during times of rapid cell growth and division, as is shown by the fact that inhibitors of the component activities of CAD such as acivicin (Aoki *et al.*, 1982), phosphonacetyl-L-asparate (Swyryd *et al.*, 1974) or 3-deazauridine (Karle and Cysyk, 1984) prevent the proliferation of tumour cell lines in cultures. In addition, rapid increases in pyrimidine nucleotide biosynthesis have been measured following stimulation of quiescent cells with mitogens, e.g., addition of serum to mouse (3T6) or chick embryo fibroblasts (Smith and Buchanan, 1979), or addition of oestrogen to a hormone-dependent human breast cancer cell line (Aitken and Lippman, 1983).

The role of cyclic AMP in regulation of cell proliferation has been controversial, although recent evidence points to the conclusion that cyclic AMP is a positive regulator of growth and proliferation during G1 phase in most cell types (Boynton and Whitfield, 1983; Rozengurt, 1986). The major growth factor for fibroblasts in crude serum is PDGF, which is known to cause a rapid elevation of cyclic AMP levels in Swiss mouse 3T3 cells, probably as a result of increased prostaglandin E production (Rozengurt *et al.*, 1983). Growth factors such as EGF or PDGF are also known to increase phosphorylation of several intracellular proteins on serine residues (Cooper *et al.*, 1982; Thomas *et al.*, 1982; Holland and Hardie, 1985). It is therefore an intriguing possibility that growth factors activate pyrimidine nucleotide biosynthesis via phosphorylation of CAD at site 1, catalysed either by cyclic AMP-dependent protein kinase or by other protein kinases with similar specificity that remain to be identified. In this regard, it is interesting that the sequence of site 1 on CAD is very similar to the sequence (Arg-Leu-Ser(P)-Ser-Leu-Arg-Ala-Ser) on ribosomal protein S6 which is phosphorylated by cyclic AMP-dependent protein kinase, and which is known to be phosphorylated (along with other sites) in response to growth factors in intact cells (Wettenhall and Morgan, 1984). We also find it intriguing that the major effect of CAD phosphorylation is to relieve inhibition by UTP.

The concentrations of ATP and UTP in mammalian cells have been estimated to be 2−3 mM and 0.5−1 mM, respectively (Jones, 1980; Jackson *et al.*, 1980). Under these conditions UTP would inhibit the phosphoenzyme much less effectively than the dephosphoenzyme (Figure 4). Stimulation of 3T6 cells with serum for 30 min, at which time there is a marked elevation of nucleotide biosynthesis, does not significantly depress UTP levels, although there is a small (~2-fold) elevation of PRPP (Smith and Buchanan, 1979). Phosphorylation of CAD on stimulation of quiescent cells may allow an activation of pyrimidine nucleotide biosyntheis in anticipation of the demand for pyrimidines which would arise during later phases of nucleic acid synthesis. Increased biosynthesis would occur in concert with increased uptake of exogenous uridine, which is another early event after stimulation by growth factors (Rozengurt, 1979).

These hypotheses are readily testable. Although activation of pyrimidine nucleotide biosynthesis represents only one aspect of the pleiotypic response of quiescent cells to growth factors (Rozengurt, 1979, 1986), it does represent a simple system with which to study the mechanism of growth factor action, since the proteins involved have been purified and are well defined.

Materials and methods

UTP, PRPP and mol. wt. markers (except fatty acid synthase) were from Sigma Chemical Co., Poole, Dorset, UK. ATP was from Boehringer Mannheim, Mannheim, FRG. Trypsin was from Worthington Diagnostic Systems Inc., USA. Recrystallized bovine serum albumin and isoelectric point markers were from BDH Chemicals, Poole, Dorset, UK. [γ-^{32}P]ATP and Na[^{14}C]HCO_3 were from Amersham International, Amersham, UK. Phosphorylase kinase, glycogen synthase and the catalytic subunit of cyclic AMP-dependent protein kinase from rabbit skeletal muscle, acetyl-CoA carboxylase from rat mammary gland, and histone H1 from calf thymus were purified as described in Munday and Hardie (1984). Fatty acid synthase was purified from rabbit mammary gland as described by Hardie and Cohen (1978). The catalytic subunits of protein phosphatases-1 and -2A were purified from rabbit skeletal muscle up to and including step 5 (polylysine-Sepharose) and assayed using [^{32}P]phosphorylase *a* in the presence of 1 mM Mn^{2+} as in Resink *et al.* (1983).

Cell culture and preparation of CAD

The transformed hamster kidney cell line 165-28, which overproduces CAD, was generously provided by Dr. George Stark. The cells were grown in culture, and CAD purified, using minor modifications of the methods of Coleman *et al.* (1977). The preparation had a high specific activity (~0.3 μmol/min/mg for the glutamine-dependent carbamyl phosphate synthetase activity) and was stored at −70°C at a protein concentration of 7−15 mg/ml in 0.1 M K^+ Hepes pH 7.0 containing 1 mM dithiothreitol, 30% (v/v) dimethylsulphoxide, 5% (w/v) glycerol ('CAD storage buffer').

Assay of ammonia-dependent carbamyl phosphate synthetase

The assays (final volume 250 μl) contained 0.1 M Tris/Cl, pH 7.5, 0.1 M KCl, 7.5% (v/v) dimethylsulphoxide, 2.5% (w/v) glycerol, 1 mM dithiothreitol, 15 mM NH_4Cl, 25 mM $MgCl_2$ (unless stated otherwise) and various concentrations of ATP. Reactions were initiated with enzyme and 25 μl of Na[^{14}C]HCO_3 (0.2 M, 0.75 Ci/mol) and were stopped after 10−25 min at 37°C by the addition of 0.15 ml of 2 M NH_4Cl. The tubes were heated at 90°C for 10−20 min, during which time carbamyl phosphate generated in the assay is converted quantitatively to urea. Unreacted bicarbonate was removed as CO_2 by acidifying with 0.1 ml of 14% (w/v) perchloric acid and drying down the solution with gentle heating. The products were resuspended in 0.4 ml water and counted in 4 ml of Optiphase MP (Fisons, Loughborough, UK). Kinetic parameters were estimated by computer fitting to the equation $v/V_{max} = S^n/(K + S^n)$, where v is the initial velocity, S is the substrate concentration, K is the apparent K_m and n is the Hill coefficient.

Phosphorylation of CAD

Unless stated otherwise CAD was phosphorylated at 30°C at a concentration of 0.8−1.2 mg/ml in 25 mM Na^+ Hepes, pH 7.0, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 25 mM $MgCl_2$, 5 mM ATP with or without the catalytic subunit of cyclic AMP-dependent protein kinase [6 units/ml, units defined in Cohen *et al.* (1977)]. The components of the CAD storage buffer (see above) were also carried through into the phosphorylation reaction (diluted 10-fold). For assays of carbamyl phosphatase synthetase, the reaction was stopped by diluting 5-fold into ice-cold assay buffer containing excess EDTA: 0.2 M Tris/Cl, pH 7.5 (at 37°C), 0.2 M KCl, 15% (v/v) dimethylsulphoxide, 5% (w/v) glycerol, 1 mM dithiothreitol, 10 mM EDTA. Incorporation of phosphate into CAD was measured in reactions containing [γ-^{32}P]ATP ($1-4 \times 10^4$ c.p.m./nmol by Cerenkov counting) by precipitation in 25% (w/v) trichloroacetic acid at 0°C as described by Guy *et al.* (1981).

When comparing rates of phosphorylation of CAD with that of other substrates (Table I), the Hepes buffer was replaced with 12.5 mM Na^+ glycerophosphate, pH 7.0, and 2.5 mM $MgCl_2$ and 0.1 mM [γ-^{32}P]ATP ($2-4 \times 10^5$ c.p.m./nmol) were used. To ensure identical conditions for each substrate, the CAD storage buffer was added at 10% (v/v) to all incubations not containing CAD. The extent of phosphorylation was limited to <0.1 mol/mol, where the incorporation was proportional to protein kinase concentration. The protein kinase was diluted to various extents in the presence of bovine serum albumin (1 mg/ml) which stabilized the diluted protein kinase. Results were corrected for trace phosphorylation that occurred when some substrates were incubated with Mg-ATP in the absence of protein kinase.

Peptide analysis and isolation

CAD (14 mg) was phosphorylated at 2.12 mg/ml and 2.5 mM $MgCl_2$, 0.1 mM

[γ-^{32}P]ATP (1.12×10^5 c.p.m./nmol by Cerenkov), and the reaction stopped after 30 min by adding trichloroacetic acid (TCA) to 2% (w/v). The stoichiometry of phosphorylation was 0.7 mol/subunit (after subtracting phosphorylation in the absence of added protein kinase). The precipitate was collected by centrifugation (14 000 *g*; 2 min), washed three times with 2% (w/v) TCA, washed once with water, and resuspended by sonication in 10 ml of 0.1 M N-methylmorpholine acetate pH 7.5. Tryptic digestion was for 16 h at 37°C at a weight ratio trypsin:CAD of 1:100. The trypsin was added in three equal aliquots after 0, 2 and 4 h of digestion. The solution was dried in a vacuum concentrator (Savant Instruments) and redissolved in 1.0 ml of 1 M acetic acid.

Labelled tryptic peptides were resolved initially on a column of Sephadex G-50 Superfine (90 × 1 cm) equilibrated in 1 M acetic acid. Two major peaks were observed eluting at Ve/Vo of 1.7 (T2) and 2.0 (T1) and accounting for 29% and 50%, respectively, of radioactivity loaded on to the column. Both peaks were further purified separately by h.p.l.c. on a Vydac C_{18} column in 0.1% (v/v) trifluoroacetic acid using a linear gradient from 0 to 40% (v/v) acetonitrile. T2 was further purified by h.p.l.c. on a Vydac C_{18} column in 10 mM ammonium acetate pH 6.5 using a linear gradient from this buffer to acetonitrile.

Amino acid analysis and sequencing

Amino acids were analysed using a 'PICOTAG' system (Millipore-Waters, Harrow, Middlesex, UK). Peptides were hydrolysed *in vacuo* for 20–24 h at 110°C in 6 M HCl containing 2 mM phenol. The samples were dried, redried in ethanol:water:triethylamine (2:2:1) and derivatized with phenylisothiocyanate:ethanol:water:triethylamine (1:7:1:1). The phenylthiocarbamyl derivatives were then analysed by reversed-phase h.p.l.c.

Automated sequence analysis was carried out on a Beckman 890D sequencer using a 0.1 M Quadrol programme with a single coupling followed by a double cleavage. Polybrene (3 mg) in aqueous solution was added to the spinning cup and dried as a film. Conversion to phenythiohydantoin (PTH) amino acids was by incubation with 20% (v/v) trifluoroacetic acid, 0.02% (w/v) dithioerythritol for 8 min at 80°C. PTH-derivatives were dissolved in 0.1% (v/v) acetic acid and 30% (v/v) acetonitrile and their ^{32}P content was determined by Cerenkov counting. The PTH-derivatives were then identified using a Waters h.p.l.c. system equipped with a Dupont Zorbax ODS column equilibrated at 50°C in 78% buffer A (27.5 mM Na^+ acetate pH 4.75, 15% (v/v) acetonitrile), 22% buffer B (33 mM Na^+ acetate pH 4.75, 55% (v/v) acetonitrile). One minute after injection a gradient from 22% to 84% buffer B was initiated using curve 11 from the Waters manual.

Phosphoamino acid analysis

Protein or peptides were hydrolysed for 2 h at 110°C in 6 M HCl. Products were analysed by paper electrophoresis in acetic acid:formic acid:water (87:22:891) pH 1.9. Phosphoserine, phosphothreonine and phosphotyrosine standards were located by ninhydrin staining.

Preparation of ^{32}P-labelled CAD for protein phosphatase experiments

CAD was phosphorylated using 5 mM ATP with or without cyclic AMP-dependent protein kinase in a final volume of 500 μl for 30 min. The protein kinase and ATP were removed by precipitating CAD by adding 325 μl of a saturated solution of ammonium sulphate in 50 mM K^+ Hepes pH 7.4, 10% (w/v) glycerol, 1 mM EDTA. The precipitates were collected by centrifugation (14 000 *g*; 2 min), redissolved in 200 μl of CAD storage buffer, and gel filtered through columns (3 × 0.9 cm) of Sephadex G-50 equilibrated in the CAD storage buffer. Dephosphorylation was initiated by adding 0.5 volumes of protein phosphatase in 50 mM Tris-HCl pH 7.0 (25°C), 50% (w/v) glycerol.

Analysis of peptides by isoelectric focussing

^{32}P-Labelled CAD was digested with trypsin as described above. Digests were dried, redried from water, and then analysed by isoelectric focussing on thin layer polyacrylamide gel using pH 3.5 to 9.5 PAG plates (LKB, Croydon, Surrey, UK) in an LKB Multiphor apparatus. After focussing, the gel was dried onto its plastic backing using gentle heat from a hair dryer. The dried gel was exposed to Kodak X-omat S film for 48–96 h at −70°C in a Kodak X-omatic cassette. Autoradiograms were scanned using an LKB laser densitometer, and an LKB Gelscan integrating programme on an Apple IIe computer was used to determine the proportion of radioactivity detected recovered in peptides T1 and T2. Purified peptides T1 and T2 were run as markers. The pH gradient was estimated using Isoelectric Point Markers (BDH Chemicals, Poole, Dorset, UK).

Polyacrylamide gel electrophoresis

This was carried out in 1.5 mm thick slab gels according to Laemmli (1970) with a 3% stacking gel and a linear 5–12.5% gradient separating gel. Gels were fixed overnight in 50% methanol, 10% acetic acid before staining with Coomassie Blue and destaining in 10% methanol, 10% acetic acid. Destained gels were dried between cellophane sheets and autoradiography was carried out as for isoelectric focussing.

Alkali-labile phosphate and protein determination

The alkali-labile phosphate content of protein preparations was determined as described by Guy *et al.* (1981). For peptides, the initial precipitation with trichloroacetic acid and washing were omitted and the dried peptide was taken up directly in 1 M NaOH for hydrolysis. The protein concentration of CAD preparations was determined by refractometry in a Spinco Model E analytical ultracentrifuge (Babul and Stellwagen, 1969) or by the dye binding method of Bradford (1976) using bovine serum albumin (recrystallized; $A^{1\%}_{280\ nm} = 6.5$) as standard. These methods agreed to within 5%. The absorbance index, $A^{1\%}_{280\ nm}$, of purified CAD was 13.0.

Acknowledgements

We thank Philip Cohen for advice and encouragement during the course of the work and preparation of the manuscript, and Brian Burchell for loaning cell culture facilities. The phosphorylase kinase, glycogen synthase and protein phosphatases-1 and -2A were gifts from colleagues in the MRC Protein Phosphorylation Group (C.McGowan, C.Smythe, T.Haystead and D.Carling). We are also very grateful to George Stark and David Evans for help with the establishment of the 165-28 cell line. This research was supported by a Project Grant (to E.A.C.) and Research Group support (to D.G.H.) from the Medical Research Council.

References

Aitken,S.C. and Lippman,M.E. (1983) *Cancer Res.*, **43**, 4681-4690.
Aoki,T., Sebold,J. and Weber,G. (1982) *Biochem. Pharmacol.*, **31**, 927-932.
Babul,J. and Stellwagen,E. (1969) *Anal. Biochem.*, **28**, 216-221.
Boynton,A.L. and Whitfield,J.F. (1983) *Adv. Cyclic Nucleotide Res.*, **15**, 193-294.
Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248-254.
Cohen,P. (1985) *BioEssays*, **2**, 63-68.
Cohen,P., Nimmo,G.A. and Antoniw,J. (1977) *Biochem. J.*, **162**, 435-444.
Cohen,P., Watson,D.C. and Dixon,G.H. (1975) *Eur. J. Biochem.*, **51**, 79-92.
Coleman,P.F., Suttle,D.P. and Stark,G.R. (1977) *J. Biol. Chem.*, **252**, 6379-6385.
Cooper,J.A., Bowen-Pope,D.F., Raines,E., Ross,R. and Hunter,T. (1982) *Cell*, **31**, 263-273.
Evans,D.R. (1986) in Hardie,D.G. and Coggins,J.R. (eds.), *Multidomain Proteins – Structure and Evolution*, Elsevier Biomedical Press, Amsterdam, in press.
Guy,P.S., Cohen,P. and Hardie,D.G. (1981) *Eur. J. Biochem.*, **124**, 399-405.
Hardie,D.G. and Cohen,P. (1978) *Eur. J. Biochem.*, **92**, 25-34.
Heldin,C.H. and Westermark,B. (1984) *Cell*, **37**, 9-20.
Hemmings,B.A., Yellowlees,D., Kernohan,J.C. and Cohen,P. (1981) *Eur. J. Biochem.*, **119**, 443-451.
Holland,R. and Hardie,D.G. (1985) *FEBS Lett.*, **181**, 308-312.
Holland,R., Witters,L.A. and Hardie,D.G. (1984) *Eur. J. Biochem.*, **140**, 325-333.
Holland,R., Hardie,D.G., Clegg,R.A. and Zammit,V.A. (1985) *Biochem. J.*, **226**, 139-145.
Jackson,R.C., Lui,M.S., Boritzki,T.J., Morris,H.P. and Weber,G. (1980) *Cancer Res.*, **40**, 1286-1291.
Jones,M.E. (1980) *Annu. Rev. Biochem.*, **49**, 253-279.
Karle,J.M. and Cysyk,R.L. (1984) *Biochem. Pharmacol.*, **33**, 3739-3742.
Laemmli,U.K. (1970) *Nature*, **227**, 680-685.
Munday,M.R. and Hardie,D.G. (1984) *Eur. J. Biochem.*, **141**, 617-627.
Otsuki,T., Mori,M. and Tatibana,M. (1981) *J. Biochem.*, **89**, 1367-1374.
Proud,C.G., Nimmo,H.G., Yeaman,S.J. and Cohen,P. (1977) *FEBS Lett.*, **80**, 435-447.
Raushel,F.M., Anderson,P.M. and Villafranca,J.J. (1978) *Biochemistry (Wash.)*, **17**, 5587-5591.
Resink,T.J., Hemmings,B.A., Tung,H.Y.L. and Cohen,P. (1983) *Eur. J. Biochem.*, **133**, 455-461.
Rozengurt,E. (1979) in Sato,G.H. and Ross,R. (eds.), *Hormones and Cell Culture, Cold Spring Harbor Conferences on Cell Proliferation*, Vol. **6**, Cold Spring Harbor Laboratory Press, NY, pp. 773-788.
Rozengurt,E. (1986) in Cohen,P. and Houslay,M. (eds.), *Molecular Aspects of Cellular Regulation*, vol. **4**, Elsevier, Amsterdam, in press.
Rozengurt,E., Stroobant,P., Waterfield,M.D., Deuel,T.D. and Keehan,M. (1983) *Cell*, **34**, 265-272.
Smith,M.L. and Buchanan,J.M .(1979) in Sato,D.G. and Ross,R. (eds.), *Hormones and Cell Culture, Cold Spring Harbor Conferences on Cell Proliferation*, Vol. **6**, Cold Spring Harbor Laboratory Press, NY, pp. 789-803.
Swyryd,E.A., Seaver,S.S. and Stark,G.R. (1974) *J. Biol. Chem.*, **249**, 6945-6950.
Tatibana,A.M. and Shigesada,K. (1972) *J. Biochem.*, **72**, 549-560.
Thomas,G., Martin-Perez,J., Siegmann,M. and Otto,A.M. (1982) *Cell*, **30**, 235-242.
Wettenhall,R.E.H. and Morgan,F.J. (1984) *J. Biol. Chem.*, **259**, 2084-2091.

Received on 4 October 1985; revised on 23 October 1985