CAD, the key enzyme in pyrimidine biosynthesis

The first step dedicated to pyrimidine biosynthesis in mammals is catalysed by carbamoyl-phosphate synthase II (CPS II; EC 6.3.5.5). This activity is regulated through feedback inhibition by UTP, the end-product of the pathway, and activated by phosphoribosylpyrophosphate (P-Rib-PP), a substrate of a later step in the pathway [1]. The CPS II activity is part of the 240 kDa multienzyme polypeptide CAD, named after the activities it contains in discretely folded domains: CPS II, aspartate transcarbamoylase (ATCase; EC 2.1.3.2) and dihydro-orotase (EC 3.5.2.3).

Phosphorylation of CAD

Protein kinase A (PKA; cyclic AMP-dependent protein kinase) phosphorylates CAD in vitro at two sites [2]. We have mapped site 1 to the C-terminal region of the CPS II domains (Figure 1), and site 2 to the linker between the ATCase and DHOase domains [3]. We believe that site 1 is the regulatory site, because the stoichiometry of labelling of site 1 is more directly related to the properties of the phosphorylated CAD, which are as follows: an increased affinity of CPS II for ATP and magnesium ions; an antagonism of the effect of UTP on CPS II; increased susceptibility of CAD to proteolytic enzymes [3].

Allosteric effectors

The antagonism between the effects of UTP and phosphorylation may result from direct steric interference between the bound UTP and phosphorylation site 1. The presence of UTP prevents phosphorylation of CAD, but not peptide substrates, by PKA [4]; thus UTP is acting upon the substrate protein to diminish the accessibility of the sites to PKA. It has been proposed that the 20 kDa C-terminal region of the CPS II domains in CAD (Figure 2) is an allosteric domain, by analogy to other CPS enzymes [5] and because the UTP effect is lost when the region is deleted [6]. However, previous work (E. A. Carrey, unpublished work) indicates that the phosphorylated CAD can still bind UTP, so it is possible that phosphorylation prevents the effect of UTP, not its binding. We are currently isolating a peptide from the allosteric domain of CAD.
Figure 2
Domains and phosphorylation sites in mammalian CAD

The functional domains are shown as stylized circles approximately in proportion to the sizes of fragments liberated by limited proteolysis. GAT, glutamine amidotransferase; CPS, duplicated ATP-binding synthase domains; 20K, domain of unknown function; allo, putative allosteric domain; DHO, dihydro-orotase domain; ATCase domain. The inter-domain linker region is shown as an exposed polypeptide chain. The putative site for binding UTP is indicated in the allo domain. The phosphorylation sites are indicated as follows: A1, A2, sites phosphorylated by PKA; D1, sequence homologous to a site for DNA-dependent protein kinase.

Figure 3
Putative sites for the regulation of key enzymes in purine biosynthesis

The similar sequences implicated in P-Rib-PP binding are shown for a number of phosphoribosyltransferases (PRTases) [14, 17, 18] and a human P-Rib-PP synthase [21], and compared with the consensus sequence for phosphorylation by AMP-PK [16]. The R residue may also be K and T may also be S; the Φ symbol represents non-polar residues such as M, I, L and V. The putative site for phosphorylation in the PAP 39 protein is also shown [22].

<table>
<thead>
<tr>
<th>P-Rib-PP synthase III (human)</th>
<th>[R] DRVAILVDQADTVTICLAAAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uracil PRTase (E. coli)</td>
<td>[R] KHKALIVPRLATGQGV</td>
</tr>
<tr>
<td>Orotate PRTase (human)</td>
<td>[R] GYCLILIDANNVQSSVL</td>
</tr>
<tr>
<td>HGPRPTase (human)</td>
<td>[R] GKVVLIVMDTIPQTMQ</td>
</tr>
<tr>
<td>Ade PRTase (human)</td>
<td>[R] GQRVVYDDDLATQGTMW</td>
</tr>
<tr>
<td>Gln amidoPRTase (rat)</td>
<td>[R] GKRVLIDSTQHTPSPIK</td>
</tr>
<tr>
<td>AMP-PK consensus</td>
<td>[R] @SXRTXIX@X@X@X</td>
</tr>
<tr>
<td>PAP 39 insert</td>
<td>[R] PDADGRHPVWYKHATVOP</td>
</tr>
</tbody>
</table>

to which UTP is covalently bound and attempting to obtain the sequence.

The activator P-Rib-PP activates CPS II by increasing the affinity for ATP and magnesium, similarly to phosphorylation. The P-Rib-PP site will be more difficult to isolate chemically because of the lability of the reagent. The consensus P-Rib-PP binding site that is seen in phosphoribosyltransferases (Figure 3) is not found in the CAD sequence.

Allostery in CPS II and ‘reciprocal allostery’ in CAD

We have proposed that the allosteric effectors and phosphorylation have a common mechanism in altering the distribution of CPS II between two states or conformational forms. The P state is stabilized by P-Rib-PP or by phosphorylation, and has a high $V_{max}$ and a low $K_m$ for ATP and for magnesium ions. The U state is stabilized by binding of UTP, and has a low $V_{max}$ and high $K_m$ for ATP and magnesium [7]. These two states resemble the R and T states respectively of classical allosteric theory. The CPS structure includes two duplicated domains with substantial sequence identity, each catalysing a different ATP-dependent part-reaction. The allosteric transition may thus entail altered interactions between the two ATP binding domains within the CPS II region rather than between two or more separate molecules of CAD. This interpretation is implicit in the model for the CPS structure, which places the allosteric domain in contact with each of the ATP-binding domains [5].

The presence of the three different enzyme activities in the multienzyme polypeptide poses some risks to the organism, notably the possibility of losing all three enzyme activities through a single polar mutation. On the other hand, the potential for enhanced activity or novel sites for regulation has often been speculated upon. We have recently proposed a novel regulatory mechanism that justifies the multienzyme structure for the CPS II and ATCase reactions. We have called the phenomenon ‘reciprocal allostery’, since the substrates for ATCase and CPS II each activate the other activity (H. S. Irvine, S. M. Shaw and E. A. Carrey, unpublished work).

We measured the incorporation by purified CAD of radioactive bicarbonate into carbamoyl aspartate or carbamoyl phosphate, and thus demonstrated the inhibition of carbamoyl phosphate
synthesis in the presence of excess unlabelled carbamoyl phosphate (product-inhibition). The overall synthesis of carbamoyl aspartate was measured by a colorimetric assay. Two phenomena indicate reciprocal interactions between the folded CPS II and ATCase domains of the multienzyme polypeptide. First, even in the presence of approx. 1 mM unlabelled carbamoyl phosphate, endogenous carbamoyl phosphate is favoured for the synthesis of radiolabelled carbamoyl aspartate when CPS II is in the liganded or P state. That is, the binding of aspartate to ATCase protects the active site of the liganded form of CPS II from inhibition by its product, and ensures that the carbamoyl phosphate is channelled to the ATCase active site. Secondly, when substrates (glutamine, ATP-Mg and bicarbonate) bind to the U state of CPS II, they act allosterically to increase the affinity of the ATCase for carbamoyl phosphate and for aspartate. When P-Rib-PP is present with low concentrations of ATP and magnesium ions, conditions that would be met during active biosynthesis in the cell, both effects operate, to protect the CPS II from product-inhibition and to channel the carbamoyl phosphate to a high-affinity site in ATCase. Similar 'reciprocal allosteric regulation' of the active sites is seen in the increased affinity of the synthase domains of CPS II for ammonia when glutamine binds to the N-terminal glutamine amidotransferase domain of CAD; the glutamine is the source of the ammonia, and the allosteric response to its binding ensures that ammonia is used efficiently by the synthase domains. The mechanism ensures that carbamoyl phosphate is efficiently synthesized and is dedicated to the second step of pyrimidine biosynthesis.

**Regulation by phosphorylation in vivo**

The phosphorylated CAD that is obtained from cell extracts exposed to radioactive ATP is labelled at sites 1 and 2, suggesting that PKA is the physiological kinase. However, substantial labelling still takes place in the presence of the PKA inhibitor peptide, and CAD has also been shown to be a substrate for S6 (ribosomal protein) kinase, whose specificity is similar to that of PKA (E. A. Carrey, unpublished work). Figure 1 compares the sites in CAD [9] with the typical target sequence that is phosphorylated by PKA. Similar sequences are found in the homologous enzymes in yeast, slime mould and fruit fly [10–12], as shown in Figure 1. The CPS II activity in the *Saccharomyces cerevisiae* URA 2 protein is not regulated by phosphorylation [10]; the labelled site corresponds not to site 1 but to site 2 in mammalian CAD.

We have identified a short CAD sequence resembling the site in p53 that is labelled by the DNA-dependent protein kinase [13]. CAD is phosphorylated *in vitro* by this kinase (S. Jackson, personal communication). The putative site (shown in Figure 2) is in the 20 kDa domain in the N-terminal half of the CPS II region of CAD, a domain to which no function has so far been ascribed. The usual substrates for this kinase are transcription factors, and phosphorylation is usually favoured by the presence of double-strand breaks in DNA, suggesting a role in DNA repair. If CAD is a genuine target for the DNA-dependent protein kinase, its role might be rationalized as a supplier of nucleotides for DNA repair, and the kinase might regulate the enzyme by tethering it close to the area that requires the nucleotides.

**Regulation of purine biosynthesis by phosphorylation**

The symmetry of the pathways for the biosynthesis of purines and pyrimidines, and the requirement for approximately equal amounts of the nucleotides in the cell, might lead us to look for more enzymes that are regulated by phosphorylation in response to external signals.

The first step dedicated to purine biosynthesis is catalysed by glutamine phosphoribosylpyrophosphate amidotransferase (amidophosphoribosyltransferase; EC 2.4.2.14), which is known to be inhibited by AMP, the end-product of the pathway. The suggestion that this enzyme is phosphorylated by PKA [14] is not borne out by the sequence [14,15], which contains no sites corresponding to the well known consensus sequence for this kinase. Intriguingly, the sequence that corresponds to a binding site for P-Rib-PP is overlapped by a consensus sequence for the AMP-activated kinase (AMP-PK), as shown in Figure 3 [16]. We have not demonstrated that glutamine P-Rib-PP amidotransferase is a substrate for AMP-PK, but it is an attractive possibility for two reasons. First, the closeness of the phosphorylation site to the position at which P-Rib-PP binds immediately suggests that the phosphorylated enzyme will be inactive because it will be unable to bind its substrate. In the crystallographic structure of the similar enzyme from *B. subtilis* [19], the P-Rib-
PP site can be seen overlapping a site at which AMP binds, inhibiting the binding of P-Rib-PP. Perhaps the phosphorylation by AMP-PK in mammals replaces the direct binding of AMP. Secondly, the AMP-PK is activated, as its name suggests, by AMP, and phosphorylation inactivates the key enzymes in the biosynthesis of fatty acids and cholesterol. A similar inactivation of purine biosynthesis would be a logical response to a rise in AMP concentrations, or to any of the stressful conditions that are implicated in the control of AMP-PK [20].

An inactivation of glutamine P-Rib-PP amidotransferase alone might lead to an increase in the concentration of P-Rib-PP in the cell, and hence to increased production of pyrimidines through activation of CPS II. The enzyme that synthesizes P-Rib-PP (P-Rib-PP synthase) has at least three mammalian isoenzymes, containing the P-Rib-PP binding consensus. An overlapping AMP-PK site is not found in these sequences. An inhibitory P-Rib-PP synthase-associated protein (PAP39) has recently been described [22] which has substantial sequence identity with P-Rib-PP synthase, but no enzyme activity. An inserted non-homologous sequence in PAP39 contains a consensus site for AMP-PK, and the protein is indeed phosphorylated by the kinase (S. Ishijima and M. Tatibana, personal communication). Our proposed mechanism here would involve the enhancement by phosphorylation of the ability of PAP39 to inhibit the activity of P-Rib-PP synthase, or perhaps the targeting of PAP to the enzyme after phosphorylation. Inactivation of the synthesis of P-Rib-PP by AMP-PK would have a fundamental role in shutting down the de novo biosynthesis of purines and pyrimidines, and the recycling of nucleotides by phosphoribosyltransferases.

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8 Reference deleted

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