FOR THE RECORD

Regulation and crystallization of phosphorylated and dephosphorylated forms of truncated dimeric phenylalanine hydroxylase

BOSTJAN KOBE,^{1*} IAN G. JENNINGS,^{2*} COLIN M. HOUSE,^{1*} SUSANNE C. FEIL,^{1*}
BELINDA J. MICHELL,¹ TONY TIGANIS,¹ MICHAEL W. PARKER,¹ RICHARD G.H. COTTON,²
AND BRUCE E. KEMP¹

¹St. Vincent's Institute of Medical Research, 41 Victoria Parade, Fitzroy, Victoria 3065, Australia

²Mutation Research Centre, 41 Victoria Parade, Fitzroy, Victoria 3065, Australia

(RECEIVED January 21, 1997; ACCEPTED March 6, 1997)

Abstract: Phenylalanine hydroxylase is regulated in a complex manner, including activation by phosphorylation. It is normally found as an equilibrium of dimeric and tetrameric species, with the tetramer thought to be the active form. We converted the protein to the dimeric form by deleting the C-terminal 24 residues and show that the truncated protein remains active and regulated by phosphorylation. This indicates that changes in the tetrameric quaternary structure of phenylalanine hydroxylase are not required for enzyme activation. Truncation also facilitates crystallization of both phosphorylated and dephosphorylated forms of the enzyme.

Keywords: crystallization; phenylalanine hydroxylase; protein phosphorylation; quaternary structure; X-ray crystallography

Posttranslational modification of proteins by phosphorylation is the most abundant form of cellular regulation, but the structural basis of its effects on proteins is poorly understood (Johnson & Barford, 1993). To understand the regulation at a molecular level, the knowledge of the three-dimensional structures of both the phosphorylated and the nonphosphorylated forms is required. At present, this is the case for only a few systems, such as glycogen phosphorylase, isocitrate dehydrogenase, and cyclin-dependent protein kinase-2. Most significantly, all these enzymes including the homologous rabbit muscle and yeast glycogen phosphorylases ex-

hibit very different control mechanisms. In rabbit muscle glycogen phosphorylase, the activation by phosphorylation occurs by an allosteric mechanism; the site of phosphorylation is more than 30 Å away from the catalytic site and elicits its effects through changes in tertiary and quaternary structure (Sprang et al., 1988; Barford & Johnson, 1989; Barford et al., 1991). In yeast glycogen phosphorylase, phosphorylation causes a rearrangement of the N-terminus and affects the arrangement of dimers; the phosphorylated threonine binds to a site analogous to the binding site of the allosteric activator AMP in mammalian glycogen phosphorylase (Rath & Fletterick, 1994; Lin et al., 1996). *Escherichia coli* isocitrate dehydrogenase is inhibited by phosphorylation through an electrostatic steric blocking mechanism; the phosphate occupies the position of a carboxyl group of the substrate citrate and prevents its binding (Dean & Koshland, 1991; Hurley et al., 1991). In CDK2, the phosphorylation causes a 7-Å movement of a loop that creates a competent substrate binding site (De Bondt et al., 1993; Jeffrey et al., 1995; Russo et al., 1996).

An important enzyme regulated by phosphorylation is phenylalanine hydroxylase (phenylalanine 4-monooxygenase, EC 1.14.16.1) (Kaufman, 1993; Hufton et al., 1995). PAH catalyzes the hydroxylation of phenylalanine to tyrosine and is the rate-limiting step in the only metabolic pathway to catabolyze phenylalanine. The reaction requires tetrahydrobiopterin as a cofactor, enzyme-bound iron, and molecular oxygen (Lazarus et al., 1981). Mutations in the PAH gene result in phenylketonuria (PKU), a common disorder in human populations that can result in severe mental retardation if untreated (Jervis, 1947; Scriver et al., 1994). Because phenylalanine is both an essential amino acid and is toxic via its metabolites, the enzyme must be tightly regulated in vivo. This is achieved by a variety of mechanisms, including phosphorylation (Abita et al., 1976; Donlon & Kaufman, 1978), activation by its substrate phenylalanine (Shiman & Gray, 1980), and regulation at a transcriptional level (Chiappelli et al., 1981; Dahl & Mercer, 1986; Konecki et al., 1992). The site of phosphorylation was identified as Ser-16 in the rat sequence (Wretborn et al., 1980).

Reprint requests to: Bostjan Kobe, St. Vincent's Institute of Medical Research, 41 Victoria Parade, Fitzroy, Victoria 3065, Australia; e-mail: b.kobe@medicine_stvin.unimelb.edu.au.

*The first four authors contributed equally to the work.

Abbreviations: 6MPH₄, 6-methyl-5,6,7,8-tetrahydropterin; BH₄, (6R)-5,6,7,8-tetrahydrobiopterin; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CDK2, cyclin-dependent protein kinase-2; DEAE, diethylaminoethyl; EGTA, ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid; MES, 2-[N-morpholino]ethane-sulfonic acid; PAH, phenylalanine hydroxylase; PEG, polyethylene glycol; TFA, trifluoroacetic acid; Tris, Tris[hydroxymethyl]aminoethane; TrpOH, tryptophan hydroxylase; TyrOH, tyrosine hydroxylase.

PAH shares many structural and catalytic properties with other members of the aromatic amino acid hydroxylases family, tyrosine and tryptophan hydroxylases, involved in the biosynthesis of catecholamines and serotonin, respectively (Hufton et al., 1995). No information is yet available on the three-dimensional structures of any of these proteins. Sequence analyses show the highest degree of conservation in the catalytic domain representing the C-terminal two thirds of the protein and highest divergence in the N-terminal regulatory domains (Hufton et al., 1995). The three aromatic amino acid hydroxylases differ markedly in their mechanisms of regulation, reflecting different physiological roles.

PAH has been reported to occur as either an equilibrium of dimeric and tetrameric forms (Kaufman & Fisher, 1970; Iwaki et al., 1986; Parniak, 1987) or solely as a tetramer (Shiman et al., 1979; Nakata & Fujisawa, 1980). TyrOH and TrpOH are found almost exclusively as tetramers (Tong & Kaufman, 1975; Markey et al., 1980; Nakata & Fujisawa, 1982). The sequences of the three aromatic amino acid hydroxylases suggested a conserved motif at the extreme C-terminus, with characteristics of coiled coils (Liu & Vrana, 1991). Subsequently, this region was shown to be required for tetramerization of TyrOH (Vrana et al., 1996) and PAH (S.E. Hufton, I.G. Jennings, & R.G.H. Cotton, unpubl.). The truncated, dimeric PAH-24, lacking the C-terminal 24 residues, has been shown by gel filtration to exist solely as a dimer, to be enzymatically active, and to be activated by the substrate, phenylalanine (S.E. Hufton, I.G. Jennings, & R.G.H. Cotton, unpubl.).

In this manuscript, we show that a dimeric PAH lacking the C-terminal 24 residues (PAH-24) remains regulated by phosphorylation. We crystallized the truncated protein in both phosphorylated and dephosphorylated states; the crystals diffract to better than 2.2 Å resolution and are suitable for the determination of the crystal structure by X-ray crystallography. The structures will explain the structural basis of enzyme regulation by phosphorylation, the structural basis of mutations resulting in PKU, and provide insight into the structure/function relationships of aromatic amino acid hydroxylases.

Results and discussion: Characterization of expressed proteins:

Proteins were expressed in insect cells and purified to homogeneity as analyzed by SDS/PAGE and mass spectrometry (Fig. 1). Masses determined by mass spectrometry corresponded to N-acetylated proteins lacking the N-terminal methionine (Aitken, 1992). The proteins were fully phosphorylated or dephosphorylated (Fig. 2).

Previously, Abita et al. (1976) found that phosphorylation activated purified rat liver PAH only when BH_4 was the cofactor. We show that the baculovirus-expressed full-length rat PAH behaves the same way, and is activated by phosphorylation only when the cofactor is BH_4 (2.5-fold increase in k_{cat}), but not when the cofactor is 6MPH_4 (Table 1). The extent of the activation is consistent with previous studies (Døskeland et al., 1984). By contrast, dimeric PAH-24, lacking the C-terminal 24 residues, is activated by phosphorylation with either BH_4 (3.5-fold) as the cofactor or 6MPH_4 (2.3-fold) as the cofactor (Table 1). PAH-24 is also activated by phosphorylation with both cofactors if k_{cat}/K_m values are considered; full-length PAH, however, appears to only be activated when 6MPH_4 is the cofactor in that case. The K_m values have, to our knowledge, not been compared previously for phosphorylated and dephosphorylated forms of rat PAH. In the case of recombinant human PAH, the K_m values for BH_4 are not affected by

Fig. 1. Electrophoretic behavior of phospho- and dephospho- forms of purified PAH and PAH-24 (0.5 µg per lane). The 10% polyacrylamide gel was stained with Coomassie blue. Lane 1, phospho-PAH; lane 2, dephospho-PAH; lane 3, phospho-PAH-24; lane 4, dephospho-PAH-24. Molecular weight markers are shown on the right, with their masses in kDa.

phosphorylation (Døskeland et al., 1996). The values we obtained are similar to values reported previously for PAH purified from rat liver (Abita et al., 1984; Haavik et al., 1986; Døskeland et al., 1987). These preparations contain about 0.3 mol of phosphate per mol of PAH (Abita et al., 1976). In addition, K_m values are affected by the extent of substrate activation by phenylalanine (Døskeland et al., 1987), and consequently the assay conditions. The relatively high errors of our measurements of K_m values currently prevent us from drawing any firm conclusions and suggest further studies. This will be of interest because regulation of PAH by phosphorylation has been shown to be relevant physiologically (Donlon & Kaufman, 1978; Kaufman, 1993).

The sequence deleted in PAH-24 was predicted to constitute a coiled coil motif in all aromatic amino acid hydroxylases. The motif is more pronounced in TyrOH and TrpOH than in PAH, where there is no perfect heptad repeat of leucine residues (Liu & Vrana, 1991). Accordingly, whereas TyrOH and TrpOH exist predominantly as tetramers, PAH exists as an equilibrium of dimeric and tetrameric forms, suggesting a weaker association of tetramers. This may be significant as a regulatory property of PAH in the light of the reported substrate-induced tetramer formation (Kaufman & Fisher, 1970; Iwaki et al., 1986; Parniak, 1987). It has been suggested that the formation of the tetramer is important for the formation of a high-affinity regulatory binding site for phenylalanine (Parniak, 1987; S.E. Hufton, I.G. Jennings, and R.G.H. Cotton, unpubl.).

The preservation of activation by phosphorylation in the truncated dimeric protein indicates that phosphorylation does not act through changes in the quaternary structure of the tetrameric form. The activation could, however, be relayed through the intersubunit contacts of the dimeric species. Because the phosphorylation sensitizes PAH toward allosteric activation by phenylalanine (Døskeland et al., 1984), this suggests an interplay between substrate activation and phosphorylation that may exploit the modulation of both dimeric and tetrameric intersubunit contacts, as well as the tertiary structures of individual subunits. Overall, the results are in agreement with the proposal that the regulatory domain contains a peptide sequence that acts as an internal inhibitor of catalytic ac-

Fig. 2. Electrospray mass spectrometry of dephospho-PAH (A), phospho-PAH (B), dephospho-PAH-24 (C), and phospho-PAH-24 (D). The data were collected on a PE Sciex API III mass spectrometer. Purified, recombinant PAH and PAH-24 were phosphorylated or dephosphorylated as described in Materials and methods. Protein was prepared for mass spectrometry by precipitation in methanol/chloroform, as described in Materials and methods, and infused directly into the mass spectrometer in 0.1% (v/v) TFA, 40% (v/v) CH_3CN .

tivity, with the negative charge introduced by phosphorylation partially displacing the inhibitory peptide through electrostatic repulsion (Fisher & Kaufman, 1973; Citron et al., 1994; Kowlessur et al., 1995). As observed for PAH, the activation of the autoinhibited protein kinase twitchin by S100A1 protein is accompanied by an

increase in $V_{\max}$ values with very small changes in K_m values (Heierhorst et al., 1996). The crystal structure shows that the auto-regulatory sequence of twitchin kinase makes a variety of contacts involving both the substrate-binding and catalytic residues (Kobe et al., 1996).

Table 1. Effect of phosphorylation on PAH activity

Protein	Phospho-PAH	Dephospho-PAH	Phospho-PAH-24	Dephospho-PAH-24
6MPH₄				
<i>K_m</i> (μM)	20.6 ± 4.7	54.2 ± 15.9	47.4 ± 10.6	34.2 ± 5.6
<i>k_{cat}</i> (min ⁻¹)	128.2 ± 9.6	140.4 ± 20.9	156.3 ± 16.5	68.1 ± 4.6
BH₄				
<i>K_m</i> (μM)	12.9 ± 6.9	1.23 ± 0.88	9.60 ± 1.17	4.5 ± 1.8
<i>k_{cat}</i> (min ⁻¹)	91.2 ± 19.8	35.8 ± 1.9	24.7 ± 0.7	7.0 ± 0.5

^aThe kinetic constants of *k_{cat}* and *K_m* (for cofactor) were determined for the various forms of PAH using 6MPH₄ and BH₄ as cofactors. Reactions were performed as described in the Materials and methods. Values are expressed ± standard error.

Crystallization: Crystals of PAH from rat liver have been reported previously (Celikel et al., 1991), but no report of the structure followed. The protein crystallized was purified from rat liver as a mixture of phosphorylated and dephosphorylated species and subsequently phosphorylated by cAMP-dependent protein kinase (Celikel et al., 1991). With our recombinant protein, we could not obtain any crystals with the published crystallization conditions with either phosphorylated or dephosphorylated protein or a mixture of these species purified from the insect cells. However, we succeeded in growing crystals in modified conditions, most important being the addition of reduced glutathione. However, the crystals were repeatedly too small for diffraction analyses; despite of extensive screening of various factors affecting crystallization and attempts of micro- and macroseeding using the existing small crystals, only one preparation of full-length phosphorylated protein yielded diffraction-quality crystals. The crystals have the same symmetry (space group P4₁22 or P4₃22) and similar unit cell dimensions to those reported previously (Celikel et al., 1991) (Table 2). There is most likely one monomer in the asymmetric unit.

By contrast, we could crystallize the truncated protein PAH-24 in both phosphorylated and dephosphorylated forms. The crystals have the same symmetry as the crystals of full-length PAH, but have a superior diffraction quality (Table 2). Several observations suggest that the dimeric form may have been crystallizing selectively from the mixture of dimeric and tetrameric forms present

with the full-length PAH. First, the crystals of the dimeric PAH-24 have the same symmetry and very similar cell dimensions as the crystals of the full-length PAH. No crystals were observed in the presence of phenylalanine with full-length PAH. It seems likely that the dimers present in the full-length preparation formed the crystals rather than the crystal packing inducing the dimers of PAH-24 to form tetramers. Second, the crystals of full-length PAH were always accompanied by heavy precipitation that may represent the tetrameric species.

Our results indicate that the PAH dimeric fragment is activated by phosphorylation. The structures of the phosphorylated and dephosphorylated truncated dimeric PAH are expected to reveal the structural basis of the regulation of this protein by phosphorylation. We chose the crystals of phospho-PAH-24 to pursue structure determination with the methods of multiple isomorphous replacement and multiwavelength anomalous dispersion, because they have the highest resolution limit and grow most reproducibly. The structure of this form should facilitate the determination of structures of other forms by difference Fourier techniques or molecular replacement, including the structure of phosphorylated full-length PAH that will reveal the three-dimensional arrangement of the C-terminal 24 residues. The structures will have general implications for structure/function relationships for the aromatic amino acid hydroxylases family and will provide an opportunity to explore the structural basis of mutations resulting in PKU.

Table 2. Diffraction data collection statistics

Protein	Phospho-PAH	Phospho-PAH-24	Dephospho-PAH-24
Unit cell ^a (Å)			
<i>a</i>	56.8	56.6	56.4
<i>c</i>	302.8	300.8	299.6
Resolution (Å)			
Observations	72,029	118,107	84,893
Unique reflections	13,091	23,972	12,374
Completeness (%)	99	91	96
<i>R_{merge}</i> ^b (%)	12.3	7.1	9.5
Average <i>I</i> /σ(<i>I</i>)	12.2	16.1	14.3
Resolution (Å)			
Average <i>I</i> /σ(<i>I</i>)	2.90–2.80	2.28–2.20	2.90–2.80
	2.2	2.0	3.2

^aAll the crystals have the symmetry of the space group P4₁22 or P4₃22 and most likely one monomer per asymmetric unit.

^b*R_{merge}* = Σ_{*hkl*}[Σ_{*i*}(*I_{hkl,i}* - ⟨*I_{hkl}*⟩)]/Σ_{*hkl,i*}⟨*I_{hkl}*⟩, where *I_{hkl,i}* is the intensity of an individual measurement of the reflection with Miller indices *h*, *k*, and *l*, and ⟨*I_{hkl}*⟩ is the mean intensity of that reflection for *I* > -3σ(*I*).

Materials and methods: *Expression, purification, and kinetic analyses:* Full-length and truncated PAH-24 were expressed in *Spo-doptera frugiperda* insect cell line Sf9 by baculovirus-driven expression (Gibbs et al., 1993) and purified by chromatography on phenyl sepharose and DEAE-cellulose columns (Shiman et al., 1979). PAH enzyme activity was assayed using a reaction mix containing 100 mM potassium phosphate buffer, pH 6.8, 65 μ g/mL catalase, 1 mM phenylalanine, 6 mM dithiothreitol, and varying amounts of pterin (either 6MPH₄ or BH₄) to calculate the Michaelis constant for the cofactor (Shiman et al., 1979). In order to observe the effect of phosphorylation, PAH was added last to start the assay. PAH-24 was assayed as for PAH, except the phenylalanine concentration was increased to 2.5 mM. Kinetic constants were calculated using computer program MMplot (Fitpak V2.10).

Phosphorylation and dephosphorylation: PAH (5–10 mg at 0.5–1 mg/mL) was phosphorylated and dephosphorylated in 25 mM MES, pH 6.8, 10 mM magnesium acetate, 0.25 mM EGTA, 0.1% (w/v) Tween 80, and either the catalytic subunit of the cAMP-dependent protein kinase (50–100 μ g mouse recombinant enzyme, prepared by the method of Yonemoto et al. (1990)) and 200 μ M ATP, or alkaline phosphatase (250 Units, 80 μ g, Boehringer-Mannheim), respectively, at 30 °C. Phosphorylation was monitored by electrospray mass spectrometry and was generally complete within 60–70 min. The samples were desalted and the buffer exchanged by Sephadex-G25 size-exclusion gel chromatography, in 30 mM Tris-HCl, pH 7.25. PAH was then re-purified on DE52 anion-exchange chromatography, with a batch elution by 0.6 M KCl in 30 mM Tris-HCl, pH 7.25. The protein was diluted threefold with 30 mM Tris-HCl (pH 7.25; final concentration of KCl was 0.2 M) and concentrated on a Centricon 30. Steps subsequent to the size-exclusion chromatography (20 °C) were performed at 4 °C, and the enzyme subsequently stored frozen at –20 °C.

Mass spectrometry: Samples of PAH (30–60 μ g) were desalted and concentrated by precipitation in methanol/chloroform. PAH solution (100 μ L) was added to 400 μ L of ice-cold methanol, 100 μ L chloroform was then added, and finally 300 μ L H₂O was added to form two phases. After centrifugation, the upper phase was removed, leaving the protein at the interface. A single phase was formed by addition of 500 μ L ice-cold methanol, followed by centrifugation. The pellets were then redissolved in 10 μ L of 30% (v/v) acetic acid and diluted twofold with acetonitrile (CH₃CN). After pelleting any undissolved material, the solution was infused directly into a PE Sciex API III electrospray mass spectrometer in a steady stream of 0.1% (v/v) TFA, 40% (v/v) CH₃CN, at a flow rate of 10 μ L/min. The ion spectrum was visualized using the supplied software (Tune 2.5-FPU) and mass distributions were calculated using the Hypermass facility in MacSpec 3.3 (Sciex).

Crystallization and collection of diffraction data: For crystallization, phospho- and dephospho-PAH or a mixed population obtained directly from the insect cells were concentrated to 3–14 mg/mL in 30 mM Tris-HCl, pH 7.25, and 200 mM KCl. Crystallization conditions were screened by the hanging drop vapor diffusion technique (McPherson, 1982; Jancarik & Kim, 1991) using Linbro cell culture plates at 4 °C and 22 °C. Small and twinned

crystals appeared in drops containing 20–25% PEG 4000, pH 5.6–7, at 22 °C. After optimization of the crystallization conditions, diffraction-quality crystals of phospho-PAH were obtained in 20% PEG 4000, 100 mM sodium citrate pH 6.0, 0.25% *t*-butanol, and 10 mM free glutathione at 22 °C; drops containing 3 μ L of protein (6.85 mg/mL) and 3 μ L of precipitant solution were hung over wells of 1.0 mL of reservoir solution. Rod-shaped crystals grew within four days to maximal dimensions of 0.05 $\times$ 0.05 $\times$ 0.3 mm. Addition of glutathione reduced the twinning and improved the size of the crystals.

The diffraction data were collected from a single crystal flash frozen at 100 K (Oxford Cryosystems) on a MARRESEARCH image plate detector at the BW7B beam line at the Deutsches Elektronen Synchrotron (DESY), European Molecular Biology Laboratory Outstation, Hamburg, Germany using radiation with a wavelength of 0.862 Å, and auto-indexed and processed with the programs DENZO and SCALEPACK (Otwinowski, 1993). Prior to data collection, the crystal was transferred to a solution containing 20% PEG 4000, 100 mM sodium citrate, pH 6.0, 0.25% *t*-butanol, and 10% glycerol and flash frozen in the nitrogen stream. Unit cell dimensions and data collection statistics are shown in Table 2.

Phospho-PAH-24 and dephospho-PAH-24 were concentrated in 30 mM Tris-HCl buffer, pH 7.25, and 200 mM KCl to 11.8 and 15.6 mg/mL, respectively. Crystallization conditions were screened as for full-length PAH. Crystals of both phospho- and dephospho-PAH-24 were obtained with PEG 4000 or 8000 as the precipitant at pH values between 5.6 and 8.5, and with ammonium sulfate as the precipitant at pH values between 7 and 8.5 at 22 °C. Thus far, we could only get thin needles with ammonium sulfate as the precipitant, but could grow diffraction-quality crystals in PEG 4000. The best crystals of phospho-PAH-24 were grown in three days in 20–24% PEG 4000, 100 mM sodium citrate buffer, pH 5.6, and 0–5 mM glutathione, by mixing 2 μ L of protein solution with 2 μ L of reservoir solution and inverting it over a 0.95-mL reservoir solution. The best crystals of dephospho-PAH-24 were grown by mixing 2 μ L of protein solution with 2 μ L of reservoir solution containing 23–25% PEG 4000, 100 mM Tris-HCl, pH 8, and 0–5 mM glutathione, and inverting it over a 0.95-mL reservoir solution. Glutathione increased the frequency of nucleation.

For cryo-crystallography, the crystals were dipped in a solution containing 22% PEG 4000, 100 mM citrate buffer, pH 5.6, and 10% glycerol for phospho-PAH-24, and 24% PEG, 100 mM Tris-HCl, pH 8, and 10% glycerol for dephospho-PAH-24 and flash-frozen in the cryo stream. The diffraction data of both phospho- and dephospho- forms were collected from single crystals at 100 K (Oxford Cryosystems) with a Weissenberg camera (Sakabe, 1991) at the Photon Factory synchrotron (Tsukuba, Japan) using radiation with a wavelength of 1.0 Å, and auto-indexed and processed with the programs DENZO and SCALEPACK (Otwinowski, 1993). The crystal of phospho-PAH were soaked in a solution containing 10 mM 1,10-phenanthroline, 22% PEG 4000, and 100 mM citrate buffer, pH 5.6, for three days prior to data collection. Unit cell dimensions and data collection statistics are shown in Table 2.

Note added in proof

Knappskog et al. (Knappskog PM, Flatmark T, Aarden JM, Haavik J, Martinez A. 1996. Structure/function relationships in human phenylalanine hydroxylase. *Eur J Biochem* 242:813–821) have recently reported that the deletion mutants of human phenylalanine

hydroxylase lacking the C-terminal 24 residues form catalytically active dimers.

Acknowledgments: We thank Jamie Rossjohn and Aaron Oakley for helping with diffraction data collection. This work was supported by grants from the NHMRC to B.E. Kemp (NHMRC Research Fellow) and R.G.H.C. (NHMRC Research Fellow). B. Kobe is an Australian Research Council Fellow.

References

- Abita JP, Milstien S, Chang N, Kaufman S. 1976. In vitro activation of rat liver phenylalanine hydroxylase by phosphorylation. *J Biol Chem* 251:5310–5314.
- Abita JP, Parniak M, Kaufman S. 1984. The activation of rat liver phenylalanine hydroxylase by limited proteolysis, lysolecithin, and tocopherol phosphate. *J Biol Chem* 259:14560–14566.
- Aitken A. 1992. Structure determination of acylated proteins. In: Hooper NM, Turner AJ, eds. *Lipid modification of proteins. A practical approach*. Oxford: IRL Press. pp 63–88.
- Barford D, Hu SH, Johnson LN. 1991. Structural mechanism for glycogen phosphorylase control by phosphorylation and AMP. *J Mol Biol* 218:233–260.
- Barford D, Johnson LN. 1989. Allosteric transition of glycogen phosphorylase. *Nature* 340:609–616.
- Celikel R, Davis MD, Dai X, Kaufman S, Xuong NH. 1991. Crystallization and preliminary X-ray analysis of phenylalanine hydroxylase from rat liver. *J Mol Biol* 218:495–498.
- Chiapelli F, Haggerty DF, Lynch M, Popjak G. 1981. Translation of phenylalanine hydroxylase-specific mRNA in vitro: Evidence for pretranslational control by glucocorticoids. *Proc Natl Acad Sci USA* 78:2105–2109.
- Citron BA, Davis DM, Kaufman S. 1994. Electrostatic activation of rat phenylalanine hydroxylase. *Biochem Biophys Res Commun* 198:174–180.
- Dahl HHM, Mercer JFB. 1986. Isolation and sequence of a cDNA clone which contains the complete coding region of rat phenylalanine hydroxylase. *J Biol Chem* 261:4148–4153.
- De Bondt HL, Rosenblatt J, Jancarik J, Jones HD, Morgan DO, Kim SH. 1993. Crystal structure of cyclin-dependent kinase 2. *Nature* 363:595–602.
- Dean AM, Koshland DE Jr. 1991. Electrostatic and steric contributions to regulation at the active site of isocitrate dehydrogenase. *Science* 249:1044–1046.
- Donlon J, Kaufman S. 1978. Glucagon stimulation of rat hepatic phenylalanine hydroxylase through phosphorylation in vivo. *J Biol Chem* 253:6657–6659.
- Døskeland AP, Døskeland SO, Øgreid D, Flatmark T. 1984. The effect of ligands of phenylalanine 4-monooxygenase on the cAMP-dependent phosphorylation of the enzyme. *J Biol Chem* 259:11242–11248.
- Døskeland AP, Haavik J, Flatmark T, Døskeland SO. 1987. Modulation by pterins of the phosphorylation and phenylalanine activation of phenylalanine 4-mono-oxygenase. *Biochem J* 242:867–874.
- Døskeland AP, Martinez A, Knappskog PM, Flatmark T. 1996. Phosphorylation of recombinant human phenylalanine hydroxylase: Effect on catalytic activity, substrate activation and protection against non-specific cleavage of the fusion protein by restriction protease. *Biochem J* 313:409–414.
- Fisher DB, Kaufman S. 1973. The stimulation of rat liver phenylalanine hydroxylase by lysolecithin and α -chymotrypsin. *J Biol Chem* 248:4345–4353.
- Gibbs BS, Wojchowski D, Benkovic SJ. 1993. Expression of rat liver phenylalanine hydroxylase in insect cells and site-directed mutagenesis of putative non-heme iron-binding sites. *J Biol Chem* 268:8046–8052.
- Haavik J, Døskeland AP, Flatmark T. 1986. Stereoselective effects in the interactions of pterin cofactors with rat-liver phenylalanine 4-monooxygenase. *Eur J Biochem* 160:1–8.
- Heierhorst J, Kobe B, Feil SC, Parker MW, Benian GM, Weiss KR, Kemp BE. 1996. Ca^{2+} /S100 regulation of giant protein kinases. *Nature* 380:636–639.
- Huften SE, Jennings IG, Cotton RGH. 1995. Structure and function of the aromatic amino acid hydroxylases. *Biochem J* 311:353–366.
- Hurley JH, Dean AM, Sohl JL, Koshland DE Jr, Stroud RM. 1991. Regulation of an enzyme by phosphorylation at the active site. *Science* 249:1012–1016.
- Iwaki M, Phillips RS, Kaufman S. 1986. Proteolytic modification of the amino-terminal and carboxyl-terminal regions of rat hepatic phenylalanine hydroxylase. *J Biol Chem* 261:2051–2056.
- Jancarik J, Kim SH. 1991. Sparse matrix sampling: A screening method for crystallization of proteins. *J Appl Crystallogr* 24:409–411.
- Jeffrey PD, Russo AA, Polyak K, Gibbs E, Hurwitz G, Massague J, Pavletich NP. 1995. Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. *Nature* 376:313–320.
- Jervis JA. 1947. Studies on phenylpyruvic oligophrenia. The position of the metabolic error. *J Biol Chem* 169:651–656.
- Johnson LN, Barford D. 1993. The effects of phosphorylation on the structure and function of proteins. *Annu Rev Biophys Biomol Struct* 22:199–232.
- Kaufman S. 1993. The phenylalanine hydroxylating system. *Adv Enzymol* 67:177–264.
- Kaufman S, Fisher DB. 1970. Purification and some physical properties of phenylalanine hydroxylase from rat liver. *J Biol Chem* 245:4745–4750.
- Kobe B, Heierhorst J, Feil SC, Parker MW, Benian GM, Weiss KR, Kemp BE. 1996. Giant protein kinases: Domain interactions and structural basis of autoregulation. *EMBO J* 15:6810–6821.
- Konecki DS, Wang Y, Trefz FK, Lichter-Konecki U, Woo SLC. 1992. Structural characterization of the 5' regions of the human phenylalanine hydroxylase gene. *Proc Natl Acad Sci USA* 89:8363–8368.
- Kowlessur D, Yang XJ, Kaufman S. 1995. Further studies of the role of Ser-16 in the regulation of the activity of phenylalanine hydroxylase. *Proc Natl Acad Sci USA* 92:4743–4747.
- Lazarus RA, Dietrich RF, Wallick DE, Benkovic SJ. 1981. On the mechanism of action of phenylalanine hydroxylase. *Biochemistry* 20:6834–6841.
- Lin K, Rath VL, Dai SC, Fletterick RJ, Hwang PK. 1996. A protein phosphorylation switch at the conserved allosteric site in GP. *Science* 273:1539–1541.
- Liu X, Vrana KE. 1991. Leucine zippers and coiled-coils in the aromatic amino acids hydroxylases. *Neurochem Int* 18:27–31.
- Markey KA, Kondo S, Shenkman L, Goldstein M. 1980. Purification and characterization of tyrosine hydroxylase from a clonal pheochromocytoma cell line. *Mol Pharmacol* 17:79–84.
- McPherson A. 1982. *Preparation and analysis of protein crystals*. New York: John Wiley and Sons.
- Nakata H, Fujisawa H. 1980. Purification and characterization of phenylalanine 4-monooxygenase from rat liver. *Biochim Biophys Acta* 614:313–327.
- Nakata H, Fujisawa H. 1982. Tryptophan 5-monooxygenase from mouse mastocytoma P815. *Eur J Biochem* 124:595–601.
- Otwiński Z. 1993. Data collection and processing. In: Sawyer L, Isaacs N, Bailey S, eds. *Proceedings of the CCP4 study weekend*. Daresbury, UK: SERC, Daresbury Laboratory. pp 56–62.
- Parniak MA. 1987. Organization of the catalytic and regulatory sites of rat liver phenylalanine hydroxylase. In: Curtius H-Ch, Blau M, eds. *Unconjugated pterines and related biogenic amines*. Berlin: de Gruyter. pp 327–337.
- Rath VL, Fletterick RJ. 1994. Parallel evolution in two homologues of phosphorylase. *Nature Struct Biol* 1:681–689.
- Russo AA, Jeffrey PD, Pavletich NP. 1996. Structural basis of cyclin-dependent kinase activation by phosphorylation. *Nature Struct Biol* 3:696–700.
- Sakabe N. 1991. X-ray diffraction data collection system for modern protein crystallography with a Weissberg camera and an imaging plate using synchrotron radiation. *Nucl Instr Meth Phys Res A* 303:448–463.
- Scriver CR, Eisensmith RC, Woo SLC, Kaufman S. 1994. The hyperphenylalaninemia of man and mouse. *Annu Rev Genet* 28:141–165.
- Shiman R, Gray DW. 1980. Substrate activation of phenylalanine hydroxylase. A kinetic characterization. *J Biol Chem* 255:4793–4800.
- Shiman R, Gray DW, Pater A. 1979. A simple purification of phenylalanine hydroxylase by substrate-induced hydrophobic chromatography. *J Biol Chem* 254:11300–11306.
- Sprang SR, Acharya KR, Goldsmith EJ, Stuart DI, Varvill K, Fletterick RJ, Madsen NB, Johnson LN. 1988. Structural changes in glycogen phosphorylase induced by phosphorylation. *Nature* 336:215–221.
- Tong JH, Kaufman S. 1975. Tryptophan hydroxylase. *J Biol Chem* 250:4152–4158.
- Vrana KE, Walker SJ, Rucker P, Liu X. 1996. A carboxyl terminal leucine zipper is required for tyrosine hydroxylase tetramer formation. *J Neurochem* 63:2014–2020.
- Wretborn M, Humble E, Ragnarsson U, Engstrom L. 1980. Amino acid sequence at the phosphorylation site of rat liver phenylalanine hydroxylase and phosphorylation of a corresponding peptide. *Biochem Biophys Res Commun* 93:403–408.
- Yonemoto WM, McGlone ML, Slice LW, Taylor SS. 1990. Prokaryotic expression of catalytic subunit of adenosine cyclic monophosphate-dependent protein kinase. *Methods Enzymol* 200:581–597.