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PHOSPHORYLATION, A FACTOR CONTROLLING THE SYNTHESIS OF L-ERYTHRODIHYDROBIOPTERIN (BH_2)

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SUMMARY

The enzyme, D-erythrodihydroneopterin triphosphate synthetase from rat brain was observed to have a significantly lower specific activity than that from liver due to their degree of dephosphorylation during preparation. The brain enzyme could be phosphorylated in vitro in presence of [^{32}P]-ATP and protein kinase, resulting in an increased specific activity. Isolation of brain enzyme in presence of 0.8 M NaF allowed recovery of the enzyme phosphorylated at residue 67 (serine) as determined by a new assay for phosphate. This enzyme is present in synaptosomes and its state of phosphorylation may regulate the rate at which dihydrobiopterin, the precursor of the hydroxylase cofactor (tetrahydrobiopterin, BH_4), is synthesized by synaptosomes.

Experimental evidence recently provided insight into the pathway of biosynthesis of tetrahydrobiopterin (BH_4)(1,2), the cofactor for a number of monooxygenases. The enzymes responsible for the synthesis of quinonoid-L-erythrodihydrobiopterin (q-BH_2) from GTP are widely distributed in mammalian tissues (3). One of the enzymes, D-erythrodihydroneopterin triphosphate synthetase converts 2-amino-6(5'-triphosphoribosyl)-amino-5- or -6-formamido-6-hydroxypyrimidine (FPyd-P_3) into quinonoid-D-erythrodihydroneopterin triphosphate (q-$NPTH_2$-P_3)(4). This hitherto undescribed enzyme, a basic protein of 9177 daltons, was recently isolated from brain and liver and purified to homogeneity which enabled its sequencing for its primary structure (5,6). It was noted that the specific activity of the pure enzyme isolated from liver was about five times greater than that of the enzyme from brain tissue. This divergence was not due to any difference in their primary structure or in their immunological response to rabbit antisera against the "brain" enzyme (5). It was therefore assumed that this basic protein was a phosphoprotein in its

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most active form and served as a substrate for the protein kinase which then selectively phosphorylated serine or threonine residues of D-erythrodihydroneopterin triphosphate synthetase. Evidence is presented here for the presence, the enzymic site of phosphate and the relevance of this phosphorylation to the control of the GTP to BH_4 pathway *in vivo*.

The pure enzyme, D-erythrodihydroneopterin triphosphate synthetase was isolated from bovine, rat brain and rat liver (3) and stored at -80°C. [γ^{32}]-ATP was prepared (7) with a specific activity of 260 mCi/mmole. Protein kinase was isolated from rabbit muscle (8).

Prior to experiments on phosphorylation with [γ^{32}]-ATP, 30 µg samples of the enzyme from rat brain and liver were hydrolyzed in 3 ml of 6 N HCl for 21 h at 105°C to demonstrate the presence of phosphate (P_i). The hydrolysate was applied to a 1x13 cm Dowex 50(-x8) column and washed with 5 ml water. The eluate and water wash were combined and lyophilized. Overall recovery of P_i was 88.2%.

Lyophilized samples were dissolved in 100 µl of water and were spotted onto a 200 µ cellulose TLC plate (Eastman) and developed for 2½ hours in sec-butanol:formic acid:water (20:5:8 v/v/v). P_i has a R_f value of 0.5, sulphate stays at the origin. After drying overnight, a 1x1 cm area around R_f 0.5 was spotted with $^{45}CaCl_2$ (carrier-free) at a molar ratio of 4:1 (4 mol $CaCl_2$/1 mol P_i expected), and air-dried. The plate was then developed three times in ethanol to a distance of at least twice the distance of the R_f of P_i in order to move the unreacted $^{45}CaCl_2$ to the ethanol front. A 1x1 cm square below, at and above the R_f of P_i was then cut out, placed in Monophase 40 and counted. In all cases, the 1x1 cm area in front of the P_i was used as the "blank". All "sample" readings were corrected by the blank. The values obtained by this new technique for quantification of P_i were linear between 0.5-24 nmoles with a 1.8% standard error of estimate.

The enzyme prepared from brain possessed some salient differences in its kinetic properties from that of liver (Table 1). Difference in the phosphate

TABLE 1: SOME CHARACTERISTICS OF D-ERYTHRODIHYDRONEOPTERIN TRIPHOSPHATE SYNTHETASE

Parameters	Source of Enzyme		
	Brain	Brain*	Liver
K_M (for FPyd-P_3)	1.4×10^{-6} M	8.9×10^{-6} M	6.5×10^{-6} M
V_{max} (nmol/mg/h)	10.0	93.5	68.0
Phosphate (mol/mol enzyme)	0.008	0.93	0.88

*NaF (0.8 M) was present throughout the purification of the enzyme.

content was particularly marked. It was assumed, therefore, that upon disruption of brain tissue, active dephosphorylation of proteins has taken place. Presence of fluoride ion, (NaF 0.8 M) a known inhibitor of phosphatases, throughout the purification of the enzyme (3,5) from brain resulted in isolation of pure D-erythrodihydroneopterin triphosphate synthetase of high specific activity (Table 1).

To confirm that the high specific activity of the enzyme was related to the presence of phosphate, in three experiments samples of the cerebral enzyme of low specific activity (9.96±0.37 nmol/mg/h) were phosphorylated in vitro. The enzyme from brain (13 µg) in 0.3 ml of 0.05 M Tris acetate buffer (pH 7.5) was phosphorylated with protein kinase (1 µg) for 10 minutes at 38°C in presence of Mg^{++} (5 mM) and [γ^{32}]-ATP (100 mM). The enzyme was separated from protein kinase, P^{32} and [γ^{32}]-ATP on a 0.4x23 cm Sephacryl S-200 column (3) with water as eluant. The catalytic activities of the phosphorylated and non-phosphorylated samples were assayed for the amount of $NPTH_2$-P_3 synthesized (3). There appeared a consequent increase in the specific activity to 80.5±1.91 nmol/mg/h and covalently bound P^{32} 0.89 mol/mol enzyme. P^{32} labelled enzyme was recovered from Sephacryl S-200 column and an aliquot was digested with trypsin (0.1% solution) in 0.05 M Tris acetate pH 8.0 at a ratio of 1:5 for 60 minutes at 38°C (5). Tryptic hydrolysis of the phosphorylated enzyme and the subsequent separation of the 12 peptides by two-dimensional and one-dimen-

sional TLC were executed as described elsewhere (5). One cm^2 spots corresponding to the various peptides were cut out and placed in vials containing Monophase 40. The amount of P^{32} was assayed by scintillation counting.

Previously we have reported (3) that the enzyme contains four serine and one threonine residue. Having recently established the sequence of the enzyme (5,6), of the twelve tryptic peptides T_2, T_6, T_8 and T_{10} contained serine residues and T_4 the only threonine residue of the enzyme. Of the tryptic peptides, T_6 (Arg.Val.Ser.Glu.Val) carried the radioactive phosphate. Peptide T_6 contained 10.4% of the P^{32} by two-dimensional TLC since chloroform:methanol: 34% ammonium hydroxide (2:2:1 v/v/v) (Solvent I) hydrolyzed 80% of the organic phosphate to P_i, 83% of P^{32} was in T_6 by one-dimensional TLC in butanol:acetic acid:water (4:1:1 v/v/v) (Solvent II). The results of tryptic digestion and TLC thus established that the serine corresponding to residue 66 of the enzyme molecule is phosphorylated. The enzyme from rat brain has an additional aspartic acid (residue 7)(3,5). Consequently, it is serine (67) of the molecule which is phosphorylated. This observation further supports the concept that proximity of arginine to serine is the specific determinant to phosphorylation to serine by protein kinase[9]. 10-20 µg samples of P^{32}-labelled enzyme protein were also subjected to 10% polyacrylamide disc electrophoresis pH 4.3. Segments corresponding to a stained duplicate were cut across the length of the gel and counted. Of the total radioactivity (24,000 dpm) applied to gel, 90% (21,670 dpm) was associated with the single band represented by the enzyme protein. Phosphorylation of the enzyme from rat brain did not alter its R_m (0.47).

The evidence that D-erythrodihydroneopterin triphosphate synthetase can be specifically phosphorylated signifies a new avenue to the understanding of the controls in the biosynthesis of D-erythrodihydroneopterin triphosphate in the mammalian tissue which as a precursor plays an important role in the regulation of the synthesis of q-L-erythrodihydrobiopterin (BH_2)(4). In turn, the cellular levels of q-BH_2 and its rate of reduction by dihydropteridine

reductase are determinant factors in the availability of BH_4 for most of the monooxygenases involved in neurotransmitter synthesis.

The estimated rate of synthesis of $NPTH_2$-P_3 in vivo[2] was 0.64 nmol/mg/h at 6 µg D-erythrodihydroneopterin triphosphate/g wet weight of rat brain. Consequently, the calculated specific activity of D-erythrodihydroneopterin triphosphate synthetase reaches 101 nmol/mg/h in vivo as compared to its optimal specific activity of ca. 93 nmol/mg/h in vitro. It is apparent, therefore, that D-erythrodihydroneopterin triphosphate synthetase is present in the tissues well nigh in completely phosphorylated form. However, dynamic state may exist between its non-phosphorylated units synthesized de novo and its phosphorylated ones. Phosphorylation of this enzyme assumes particular significance in the brain since previously it was revealed that the enzymic pathway which subserves the conversion of GTP to BH_2 is operant in the synaptosomes. In terms of enzyme activity, the synaptosomal fraction in presence of NaF is capable of synthesizing about 2.4 nmol/mg/h $NPTH_2$-P_3 from FPyd-P_3 if prepared by Ficoll (10), and 0.1 nmol/mg/h by sucrose (11) density gradient. The mechanism for phosphorylation and dephosphorylation of D-erythrodihydroneopterin triphosphate synthetase is available in the synaptic membrane since the latter is geared to the formation and degradation of cyclic nucleotides. Certain proteins in the synaptosomes were reported to be phosphorylated consequent to stimulation of c-AMP synthesis (12). It is likely that phosphorylation of tryptophan and tyrosine hydroxylases also occur at the synaptosomal level, which would result in decreased K_M for BH_4 (13). This phosphorylation in vivo may require ATP and Mg^{++} only or could be c-AMP mediated. One could make a case that the phosphorylation of hydroxylases could serve as a feedback effect in regulation of the synthesis of q-BH_2 and perhaps in the control of q-dihydrobiopterin reductase activity. At any event, the phosphorylation of D-erythrodihydroneopterin triphosphate synthetase significantly altered its V_{max}. The phosphorylation of this enzyme on the pathway from GTP to q-BH_2 may not be the only one. It is conceivable that activity of L-erythrodihydro-

biopterin synthetase may also be regulated by phosphorylation. This problem and other more complex ones like phosphorylation at synaptosomal level is currently under study.

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