PN 1227

A general reaction of diisopropylphosphorofluoridate with proteins without direct effect on enzymic activities

The reaction of diisopropylphosphorofluoridate with proteins has been studied mostly from the point of view of the coupling of DFP to the enzyme active centers. Little information has been available on the alkylphosphorylation at sites other than the active center, although such a reaction was reported to occur in a few instances[1-3]. In the course of an investigation of bromelain[4,5] we have found that the enzyme is not inhibited by DFP but that it does react with DFP leading to the formation of a fully active, phosphorus-containing enzyme. Similar results have been obtained also with crystalline papain and crystalline Taka-amylase A. Furthermore, an enzymically inactive mercuri derivative of bromelain, prepared by treating bromelain with $HgCl_2$, was found to be as readily phosphorylated by DFP as the original, active bromelain. From these experimental results it is suggested that the reaction of DFP with proteins is a general type of alkylphosphorylation reaction at certain site or sites of the protein molecules which may or may not coincide with those regions of the enzyme surface where its substrate is localized and activated during its enzymic action. In fact, bovine serum albumin, a non-enzyme protein, was also shown to be readily phosphorylated by DFP under the conditions employed.

TABLE I

REACTION OF DFP WITH VARIOUS PROTEINS

The reaction was carried out at 30° (pH 8.2) for 3 h. For other experimental conditions, see text. Substrates used for enzymic assays were: casein[6] for proteases; amylose[9] for amylase; and glycol chitin[10] for lysozyme. The activity is expressed in terms of per cent of that of the untreated enzyme.

Protein	*atoms P/mole protein*	*Enzymic activity in %*
Bromelain	0.91	101*
Mercuri bromelain	0.96	98*
Papain[11]	0.80	103*
Trypsin**	0.98	0
Bacillus subtilis protease***	1.02	0
Streptomyces griseus protease§	0.90	36
Pseudomonas aeruginosa protease[12]	0.02	104
Taka-amylase A[13]	0.69§§	100
Egg-white lysozyme	0.04	100
Bovine serum albumin§§§	1.10	

* With 5 mM cysteine.
** Worthington Biochemical Corporation, Freehold, N.J.
*** Nagase Sangyo Co., Amagasaki, Japan.
§ Kaken Chemical Co., Tokyo, Japan. The reaction was carried out at pH 9.0.
§§ After 5 h incubation.
§§§ Nutritional Biochemicals Corporation, Cleveland, Ohio.

1–2 μmoles crystalline protein were incubated in 3 ml 0.25 M Tris buffer (pH 8.2) with 88 μmoles DFP at 30° for 3 h. DFP used was the product of Mann Research Laboratories, New York, N.Y., or a sample kindly supplied by Sumitomo Chemical Industries, Co., Osaka, Japan. After 3 h, the reaction mixture was fractionated on a 2.8 × 40 cm Sephadex G-25 (Pharmacia, Uppsala, Sweden) column with 0.05 M

acetate buffer (pH 5.2). The protein fraction obtained was analyzed for protein concentration as measured by the absorbancy at 280 mμ and for phosphorus content[6], and also the enzymic activity was assayed (Table I).

In order to show that the phosphorus found in the gel filtrate combines firmly to the protein molecule the following experiment was carried out with bromelain. After the gel filtration of DFP-treated enzyme, the protein was precipitated by saturation with ammonium sulfate. The precipitate was dissolved in 3 ml water and dialyzed against two changes of 2 l water for 30 h in the cold. Aliquots were then withdrawn for assays from the dialyzed solution, and with the remainder the whole procedure of precipitation, redissolution, dialysis, and the analyses was repeated. Both the phosphorus content and the enzymic activity at each step of these procedures were found to remain constant, suggesting a true alkylphosphorylation with no inhibition.

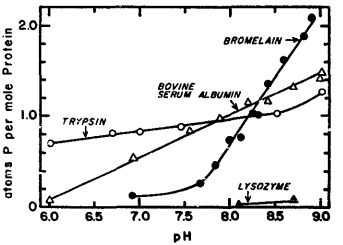

Fig. 1. Effect of pH on the alkylphosphorylation of proteins by DFP. The experimental conditions were the same as those for Table I, except that Tris–maleic acid buffer was used for pH's 6.0–7.7.

The reactions of DFP with bromelain and bovine serum albumin were found to show marked pH dependence (Fig. 1). It is interesting to note that more than one atom phosphorus per mole protein is incorporated at pH above 8.5. The reaction of trypsin with more than one equivalent of DFP has been reported previously[7]. The identification of the phosphorus-containing products obtained by partial hydrolysis of the phosphorylated bromelain and albumin are now in progress.

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PN 1221

Sur la biosynthèse *in vitro* des hormones thyroïdiennes à partir de la L-tyrosine tritiée

La 3,5-diiodo-L-tyrosine et la 3-monoiodo-L-tyrosine, considérées comme précurseurs des hormones thyroïdiennes, prennent naissance par iodation de la tyrosine et l'on admet, selon une hypothèse d'HARINGTON[1] généralisée par ROCHE *et al.*[2], que les iodothyronines sont biosynthétisées par condensation de deux molécules d'iodotyrosine avec perte d'une chaîne d'alanine. Divers arguments expérimentaux, pour la plupart indirects, ont déjà été fournis en faveur de cette hypothèse; néanmoins il nous a paru utile de vérifier si la tyrosine fixée par la glande au cours de la biosynthèse de la thyroglobuline est bien le précurseur du squelette carboné des iodothyronines; nous y sommes parvenus grâce à l'emploi de tyrosine tritiée en position $\alpha\beta$, d'activité spécifique élevée (86 mC/mmole)[3].

A. *Conditions d'incubation permettant l'hormonogénèse in vitro.* La formation de thyroxine *in vivo* ne se manifeste que 6 h environ après l'injection d'$^{131}I^-$. Elle a été rarement signalée *in vitro*; les incubations de coupes de glande n'ont été en général poursuivies que pendant quelques heures et les critères d'identification de thyroxine employés n'ont pas toujours été rigoureux. Les recherches poursuivies *in vivo* avec ^{131}I sont facilitées par la concentration thyroïdienne des iodures, mais tel ne peut être le cas pour la tyrosine, laquelle est fixée par l'ensemble des tissus après son injection. On ne pouvait donc escompter de résultats satisfaisants qu'*in vitro*, dans la mesure où la survie de coupes serait assurée pendant un temps assez long. Nous y sommes parvenus en incubant 100 mg de coupes sous O_2 à 37° pendant 24 h dans 3 ml d'un milieu nutritif approprié[4] additionné soit d'$^{131}I^-$ soit d'[^{3}H]tyrosine. Q_{O_2} (10 essais par série de mesures) demeure constant pendant toute la durée de l'incubation.

B. *Incorporation d'^{131}I et d'[^3H]tyrosine dans la thyroglobuline: identification des iodothyronines formées.* Les coupes et le milieu d'incubation sont analysés soit séparément, soit après homogénéisation et isolement des particules cellulaires (mitochondries-microsomes, surnageant) par centrifugation différentielle (700 × *g* pendant 2 min, puis 105000 × *g* pendant 1 h). Les liquides obtenus sont analysés à l'état brut ou après dialyse, par chromatographie sur papier (solvants: *n*-butanol–acide acétique–eau (78:5:17) et (8:2:2), amylol tertiaire saturé de NH_4OH 2 N) et électrophorèse sur papier (véronal pH 8.6, 18 h). Il a été ainsi établi qu'$^{131}I^-$ et [^{3}H]tyrosine sont fixés par les coupes et partiellement incorporés dans la thyroglobuline de celles-ci et qu'il se forme également d'autres protéines iodées et tritiées, de mobilité électro-