Biochimica et Biophysica Acta, 397 (1975) 519—528

BBA 67551

STRUCTURAL REQUIREMENTS FOR THE ENZYMATIC PHOSPHORYLATION OF PHOSVITIN

L.A. PINNA, A. DONELLA, G. CLARI and V. MORET

Institute of Biological Chemistry, University of Padova and "Centro per lo Studio della Fisiologia Mitocondriale" del C.N.R., Padova (Italy)

(Received December 30th, 1974)

Summary

Some structural features required for the enzymatic phosphorylation of phosvitin by purified rat liver cytosol phosvitin kinase have been investigated by testing the activity of such an enzyme toward phosphopeptides differing in size and chemical composition, obtained by pronase or acid hydrolysis of phosvitin. The results obtained can be summarized as follows:

(a) Phosvitin kinase phosphorylates even fairly simple phosphopeptides (mol.wt 1000—2000) at rates comparable with intact phosvitin.

(b) Acetylation of both phosvitin and pronase phosphopeptides completely prevents their phosphorylation indicating that some lysine residues are strictly required for the phosvitin kinase reaction.

(c) Accordingly polyphosphorylserine blocks $Ser(P)_n$ which are very actively phosphorylated in phosvitin and pronase phosphopeptides, do not undergo any more enzymatic phosphorylation once isolated as such in a form free of other amino acids.

(d) The activity of phosvitin kinase toward substrates probably devoid of $Ser(P)_n$ blocks suggests that these are not required for the protein kinase reaction. However, they apparently enhance the phosphorylation rate of the peptide substrates, likely by making easier their binding to the enzyme.

It is proposed therefore that the peptidic unit able to undergo phosphorylation by rat liver cytosol phosvitin kinase consists of one or more phosphorylserine residues having in their close proximity a lysine residue playing a critical role in the mechanism of transphosphorylation.

Introduction

"Acidic" phosphoproteins are so called to distinguish them from histones and protamines, because, unlike these latter compounds, they contain, besides phosphorylserine and phosphorylthreonine, relatively few residues of basic

Abbreviations: Ser(P), phosphorylserine; $Ser(P)_n$, polyphosphorylserine blocks.

amino acids. In spite of their large diffusion, at least in eucariotic organisms [1,2], only in few instances have acidic phosphoproteins been isolated from cell nuclei [1,3] and liver cytosol [4,5] and partially characterized. Owing to the difficulty to isolate them in a pure form and in appreciable amounts, most frequently phosvitin, the well characterized phosphoglycoprotein from egg yolk [6—9], has been used as a model substrate in the investigations concerning such a class of proteins, and the protein kinases responsible for their phosphorylation are often referred to as "phosvitin kinases" (belonging to the group of ATP:protein phosphotransferase, EC 2.7.1.37) [10].

The present paper gives some information about the substrate structural features required for such a protein kinase reaction. For this purpose the susceptibility to cytosol phosvitin kinase of phosphopeptides obtained by pronase or acid hydrolysis of phosvitin has been investigated. Particular attention has been devoted to phosphorylserine blocks $Ser(P)_n$, very abundant in phosvitin, but also present in casein [11] and in some tissues phosphoproteins [12,13], which have been suggested to be responsible for the specificity of such a protein kinase reaction [14,15]. Our results indicate that $Ser(P)_n$ runs are not strictly required. However, they are preferentially phosphorylated provided that they participate to a peptide chain having lysine residues in the environment. Once isolated in a free form they no longer undergo the kinase reaction.

Experimental

Phosvitin was prepared from egg yolk according to Mecham and Olcott [16]. Its P content was 9.1%.

Pronase digestion of phosvitin was accomplished according to Shainkin and Perlmann [8]. At the end of digestion pronase was inactivated by keeping the sample at 100°C for 3 min and the digestion products were submitted to gel chromatography through a Sephadex G-50 column (135 × 1.80 cm) equilibrated with 10% acetic acid. The pattern obtained is described in details in Results.

Phosphorylserine blocks $Ser(P)_n$ were prepared following in its general lines the procedure of Williams and Sanger [11]: for a typical preparation 100 mg of phosvitin were dissolved in 10 ml of 12 M HCl and incubated in a sealed tube for 18 h, at 37°C. HCl was removed under vacuum over P_2O_5 and NaOH, and aliquots of the hydrolysate submitted to paper electrophoresis on Whatman 3 MM in 20% formic acid, pH 1.5, at 100 V/cm for 90 min. Phosphorylated bands were evidenced in a parallel guide strip with the Hanes and Isherwood reagent [17]. The two main phosphorylated bands running faster than P_i were eluted overnight with water and characterized for their amino acid composition, P content and average molecular weight. The analytical results, reported in Table I are consistent with the previous conclusions of Williams and Sanger [11] that such bands are $Ser(P)_4$ and $Ser(P)_5$, respectively. In most experiments, however, they were eluted and mixed together.

Acetylation of phosvitin was carried out by addition of acetic anhydride to a 10% phosvitin solution in half saturated sodium acetate (see ref. 18). The pH was kept between 7 and 8 by addition of 5 M NaOH. Salts were removed by dialysis against three changes of 200-folds excess distilled water. Phosphopep-

tides P1 and P2b were acetylated by a similar procedure except dialysis was replaced by gel filtration through a Sephadex G-25 column (32 × 1.4 cm) equilibrated with 10% acetic acid. Acetylated phosphopeptides were recovered near the V_0 of the column. The occurrence and extent of acetylation were evaluated by the decrease of ninhydrin reactivity which was 60% for phosvitin and 85% for phosphopeptides.

Phosvitin kinase was purified over 1000-folds from rat liver cytosol as previously described [19]. Purification was sometimes furtherly improved by a procedure consisting in Sepharose 6B gel filtration at high ionic strength (increasing at least 5-folds the specific activity) followed by polyacrylamide gel electrophoresis. The gels were prepared and prerun following the Kish and Kleinsmith procedure [20] except the acrylamide concentration was 5%. After electrophoresis the gels were sliced into 3-mm segments which were eluted overnight with 0.5 ml of 4 mM mercaptoethanol. All phosvitin kinase activity was recovered in a band moving between 2 and 3 cm from origin.

Protein kinase activities were evaluated by the different procedures:

(A) Variable amounts of substrate proteins (phosvitin, salmine or histones type IIA from Sigma) were incubated for 10 min at 37°C in 0.5 ml of a medium containing: 200 mM Tris · HCl buffer, pH 7.5; 6 mM $MgCl_2$; 50 μM [γ-^{32}P]ATP with a specific radioactivity of about 40 Ci/mol and 5—10 μg of phosvitin kinase [19]. Incubation was stopped by the addition of 5 ml of ice-cold 10% trichloroacetic acid plus silicotungstic acid solution (9 : 1, v/v) [21]. Precipitated proteins were recovered by centrifugation and washed four times more with the same solution before being dissolved with 10 ml of "Instagel" and counted in a Packard liquid scintillator.

(B) For the determination of protein kinase activity toward phosphopeptides most of which are still soluble in the trichloroacetic acid silicotungstic acid solution, a new method was devised taking advantage of the relative acid stability of protein-bound P compared with the high acid lability of ATP. According to such a procedure, phosphopeptides (or phosphoprotein) equivalent to 0.60 μmol P were incubated at 37°C in 0.25 ml of a medium identical to that of procedure A except the specific radioactivity of [γ-^{32}P]ATP was 2—4 times higher.

The incubation was stopped after variable times by addition of HCl to make its concentration 1 M and the volume 1 ml, followed by 10 μl of unlabeled 0.10 M ATP. The samples were transferred to vials which were sealed and heated at 105°C for 7—10 h*. Under these conditions [γ-^{32}P]ATP is completely converted into $^{32}P_i$ while less than 50% of the protein-bound P is hydrolyzed. Inorganic P was now converted into the phosphomolibdic complex and removed from the water phase (4 ml) by extraction with 5 ml of isobutanol/benzene (1 : 1, v/v), according to the Martin and Doty procedure [22]. Isobutanol/benzene extraction was repeated four times more and finally 1 ml of the aqueous phase, containing ^{32}P-labeled phosphorylserine and small phosphopeptides generated during 1 M HCl hydrolysis, was counted in a Packard

* It was found preferable that hydrolysis lasted for several hours in order to convert completely the radioactive peptides into free phosphorylserine and very small phosphopeptides remaining homogeneously soluble throughout the whole procedure.

liquid scintillator. Controls were always run where either the enzyme or the substrate were omitted.

Results

(1) Breakdown of phosvitin and characterization of phosphopeptide fractions

The breakdown of phosvitin molecule into phosphorylated peptides was accomplished either by pronase digestion [8] or by 12 M HCl hydrolysis [11].

The gel filtration of pronase-digested phosvitin through Sephadex G-50 gives rise to the pattern reported in Fig. 1. By assaying total phosphate, three distinct peaks were constantly obtained: the minor one (P_3), eluted near the column volume was found to be almost completely accounted for by inorganic phosphate generated during incubation or contaminating the original material. The middle peak was usually collected in such a way to give three fractions (P2a, P2b and P2c), while the first peak (P1) was pooled as a whole (see Fig. 1). From the data reported in Table I, showing some properties of such phosphopeptidic fractions, it should be concluded that: (a) the action of pronase was exhaustive since the single methionine residue which is known to be present in phosvitin molecule [9] is recovered only in one fraction (P2c) and not in the remaining ones; (b) the pronase digestion spares phosphorylserine rows (accounting for large part of the phosvitin molecule) and their neighbourhoods. This latter point is indicated by three findings: (1) the neglegible amount of free phosphorylserine recovered in peak P3 together with other amino acids and small peptides (major peak of the ninhydrin pattern in Fig. 1); (2) the increased content of protein-bound P and serine in most phosphopeptides in comparison with phosvitin itself. It should be noted that the larger phosphopeptides of the P1 fraction can be considered as very long phosphorylserine blocks, interrupted by few different amino acid residues, namely aspartic acid, lysine and arginine. (3) pronase was found to be inactive on isolated $Ser(P)_n$ blocks, obtained by acid hydrolysis of phosvitin.

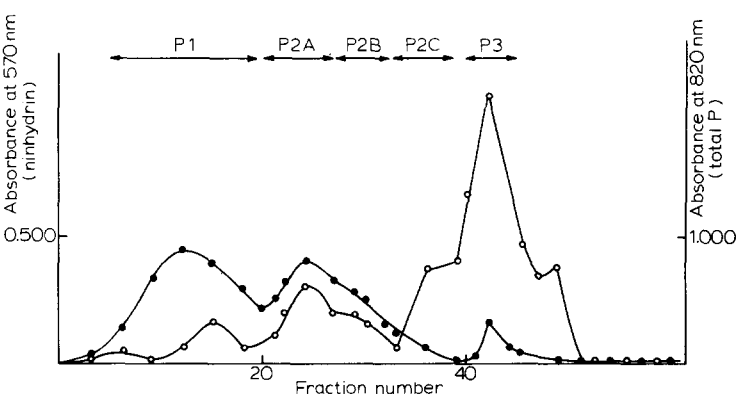

Fig. 1. Sephadex G-50 gel chromatography of pronase-digested phosvitin. Pronase digestion and gel filtration of the products were carried out as described in Experimental. The flow rate was 20 ml/h. 4-ml fractions were collected which were analyzed for total P [23] (●———●) and for ninhydrin-positive material after alkaline hydrolysis [28] (○———○).

TABLE I

CHARACTERIZATION OF PHOSPHOPEPTIDE FRACTIONS OBTAINED BY PRONASE OR ACID DIGESTION OF PHOSVITIN

Phosphopeptide fractions were prepared as described in Experimental and in the legend of Fig. 1. Amino acid analysis was carried out in a Geol Gacylc 5AH apparatus after 24 h hydrolysis of 6 M HCl at 105°C under N_2. Amino acid composition are expressed in terms of mol per 100 mol of amino acids recovered, after correction of serine content assuming that 25% of the phosphoseryl residues are destroyed by 24 h hydrolysis [6]. No attempt was made to determine tryptophan. Total P was determined by the Wagner procedure [23]. Average molecular weights were determined by gel filtration through either Sephadex G-50 (pronase peptides) or G-25 (HCl peptides) columns (0.9 X 70 cm) equilibrated with 10% acetic acid and previously calibrated with Dextran blue, cytochrome *c*, salmine, insulin A chain, RNAase synthetic octapeptide 13—20, oxidized and reduced glutathion. n.d., not determined.

Amino acid	Phosvitin	P1	P2a	P2b	P2c	Ser$(P)_n$
Asp	6.20	3.73	5.52	10.58	8.28	traces
Glu	5.78	—	1.88	0.25	13.40	traces
Thr	2.13	n.d.	4.22	2.75	5.46	traces
Ser	56.23	84.00	64.16	64.33	21.39	$\geqslant$ 90.00
Pro	1.42	—	0.49	—	1.24	—
Gly	2.43	1.45	2.86	0.24	7.64	—
Ala	3.51	0.73	5.72	3.29	6.12	traces
Val	1.44	0.34	1.30	2.13	2.77	—
Met	0.33	—	—	—	0.53	—
Ile	0.76	—	1.05	—	1.99	—
Leu	1.06	0.19	3.50	1.77	2.59	—
Tyr	0.38	—	—	—	0.24	—
Phe	0.83	—	—	—	0.60	—
Lys	7.39	5.34	3.43	7.56	14.02	traces
His	4.62	—	2.33	2.81	9.06	—
Arg	5.06	4.11	3.47	4.24	4.75	—
Total P (mol/mol serine)	0.96	0.93	0.94	0.94	0.90	0.92
Average molecular weight	$3.4 \cdot 10^4$	5800	2500	1800	700—1500	780

(2) Phosphorylation of phosphopeptides by cytosol phosvitin kinase

As shown in Fig. 2 purified phosvitin kinase phosphorylates most of pronase peptides at rates comparable with phosvitin. Even the smallest pronase peptides of P2c fraction are phosphorylated, though less actively. On the contrary no activity at all could be evidenced toward isolated Ser$(P)_n$ blocks obtained by acid hydrolysis nor toward phosphorylserine itself. It should be noted on this matter that when phosvitin is previously phosphorylated by [γ-^{32}P]ATP through the kinase reaction, all the phosphopeptide fractions of its acid hydrolysate are found to be labelled, but by far most of the radioactivity is present in the large Ser$(P)_n$ blocks fractions (see Fig. 3). It should be concluded therefore that Ser$(P)_n$ blocks, while are sites of active phosphorylation in the intact molecule of phosvitin and in fairly short fragments of it, are no more susceptible to enzymatic phosphorylation once isolated as free form.

On the other hand the Ser$(P)_n$ runs seem not to be strictly required for the protein kinase reaction, since the rather basic phosphopeptides of the P2c fraction can be phosphorylated by the enzyme although radioactive Ser$(P)_n$ runs could not be isolated from them. Such a conclusion is also supported by the finding that histones too are phosphorylated by purified phosvitin kinase,

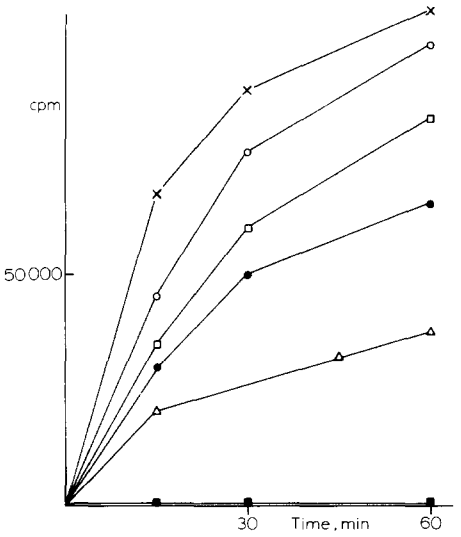

Fig. 2. Phosvitin kinase-dependent phosphorylation of phosphopeptides from phosvitin. Protein kinase activity was tested according to procedure B described in Experimental. ○———○, phosvitin; ●———●, P1; X———X, P2a; □———□, P2b; △———△, P2c; ■———■, Ser(*P*)$_n$ and phosphorylserine.

though the presence of Ser(*P*)$_n$ blocks in their molecule is quite unlike [24] (Table II).

Table II also shows that the protamine salmine, a basic phosphoprotein similar to histones under several respects but, unlike them, completely lacking of lysine [25] is not phosphorylated at all by phosvitin kinase. Moreover, acetylation of phosvitin and pronasic phosphopeptides prevents the protein kinase reaction from taking place, as shown in Table III. Such an inhibitory effect is not abolished when the acetylated substrates are previously treated with hydroxylamine under conditions which are known to remove acetyl

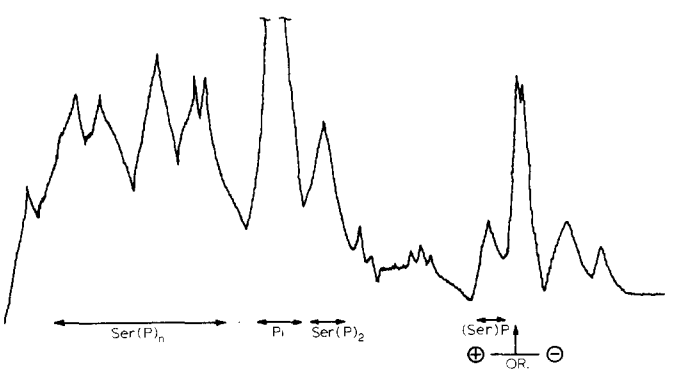

Fig. 3. Evidence for the enzymatic phosphorylation of Ser(*P*)$_n$ blocks in the intact phosvitin molecule. Phosvitin (2 mg) was labeled by 90 min incubation with phosvitin kinase and [γ-^{32}P] ATP according to method A (see Experimental). Labeled phosvitin was submitted to 12 M HCl hydrolysis followed by high voltage electrophoresis as described in Experimental for the preparation of Ser(*P*)$_n$ blocks. Radioactive areas were located by a Packard radiochromatoscanner. The specific radioactivity of the main phosphorylated compounds was determined after elution by measuring the total P content [23] and counting an aliquot of the eluate in a liquid scintillator.

TABLE II

PHOSPHORYLATION OF HISTONES BY PURIFIED CYTOSOL PHOSVITIN KINASE

Further purification of phosvitin kinase by gel electrophoresis was carried out as described in Experimental. Protein kinase was tested by method A.

Phosvitin kinase purified up to:	^{32}P incorporated into protein substrate (cpm)			cpm in phosvitin / cpm in histones
	Phosvitin (1 mg)	Histone (10 mg)	Salmine (10 mg)	
P-cellulose stage	39800	8800	190	4.4
Gel electrophoresis stage	35400	8450	78	4.2

TABLE III

INHIBITION OF PROTEIN KINASE REACTION BY ACETYLATION OF THE SUBSTRATE

General conditions for acetylation and assay of protein kinase activity are described in Experimental (procedure B). Treatment with hydroxylamine was carried out at pH 11 essentially according to ref. 29. pH was lowered to 7.5 by addition of HCl before the enzymatic test. Such a treatment was ineffective on the enzymatic phosphorylation of control phosvitin.

	^{32}P incorporated (cpm)		After acetylation and NH_2OH treatment
	Control	After acetylation	
Phosvitin	62500	6380	6890
Peptides P1	51405	5585	—
Peptides P2b	58275	6912	6720

residues from hydroxyl groups but not from lysine [26]. This indicates that the masking of some amino groups must be responsible for the unability of acetylated substrates to be phosphorylated.

On the other hand the possibility that acetylated phosvitin is still able to interact with the kinase has been checked by the kinetic experiment shown in Fig. 4 where is reported the Lineweaver-Burk double-reciprocal plot for phosvitin kinase activity obtained by using phosvitin as substrate in the presence

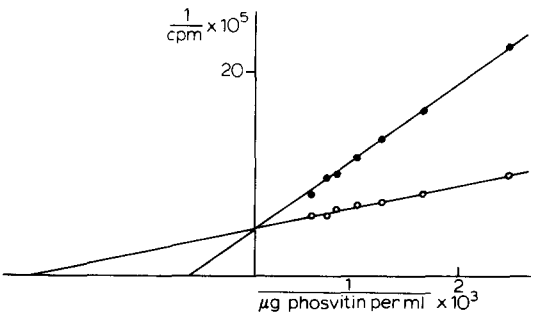

Fig. 4. Competitive inhibition of phosvitin kinase by acetylated phosvitin. Phosvitin kinase activity was determined following procedure A described in Experimental. Carrier phosvitin was added after trichloroacetic acid at the end of incubation in order to have in every test tube 1 mg phosphoprotein. ○———○, control; ●———●, plus 0.1 mg of acetylated phosvitin.

and in the absence of acetylated phosvitin. It can be seen that acetylphosvitin which can be no more phosphorylated though having all its acidic residues unmodified, behaves like a competitive inhibitor on the enzymatic phosphorylation of the non-acetylated phosvitin. This suggests that acetylated phosvitin is still able to bind, possibly through its acidic blocks, to the cathalytic site, thus preventing the binding of substrate phosvitin.

Discussion

The experiments reported in the present paper provide information about some aspects of the enzymatic phosphorylation of phosvitin by purified cytosol phosvitin kinase, a protein kinase apparently unaffected by cyclic AMP [4].

The first conclusion which can be drawn from our data is that the integrity of the phosphoprotein molecule is not required for the kinase reaction, since even phosphopeptides with molecular weight lower than 2000 can be phosphorylated at rates comparable with that of phosvitin. Moreover, it was possible to isolate by Sephadex G-25 gel filtration of the less actively phosphorylated P2c fraction labelled peptides having molecular weights in the range between 600 and 1000.

However, phosphorylserine blocks which are preferentially phosphorylated when taking part of both phosvitin and shorter phosphopeptides molecules, can no more undergo the protein kinase reaction once isolated as free form*, thus suggesting the involvement of some other structural feature of the substrate molecule for the enzymatic phosphorylation. Such a critical requirement seems to be the presence of lysine residues since the acetylation of phosvitin and phosphopeptides completely prevents the protein kinase reaction. Such a conclusion is also confirmed by the finding that phosvitin kinase is active toward histones but not salmine which is lacking of lysine residues. It should be emphasized on this matter that the activity of the enzyme toward histones is not due to contaminations by different protein kinases since the ratio between the activities toward the two substrates (phosvitin and histones) does not change upon further purification by Sepharose 6B gel filtration followed by polyacrylamide gel electrophoresis. The requirement of lysine residues for the protein kinase reaction can be explained by assuming that protein phosphorylation takes place through a two stages mechanism involving the incorporation of phosphate at lysine residues followed by its shifting to serine and threonine hydroxyles. It must be recalled that evidences for a phosphate shift between amino and hydroxyl groups of phosvitin have been reported several years ago by Taborsky and Allende [27], which also suggested the possible involvement of such a mechanism in the kinase reaction.

Finally we tried to give an answer to the problem concerning the role of the blocks of several consecutive phosphorylserine residues, typical of phosvitin, in the protein kinase reaction. Our results would indicate that such blocks

* The failure of isolated Ser$(P)_n$ blocks to undergo phosphorylation cannot be due to the lack of available serine hydroxyl sites (assuming that a net phosphorylation rather than an exchange with [^{32}P] ATP takes place) since the discrepance between serine and P content (see Table I) indicates that about 10% of the serine residues in the pooled Ser$(P)_n$ fraction are not in the phosphorylated form.

are not strictly required, since histones and P2c phosphopeptides can be phosphorylated in spite of their probable lack of Ser$(P)_n$ runs. It is likely, however, that Ser$(P)_n$ runs improve the protein kinase activity since the phosphopeptide fractions containing such blocks are better substrates than P2c which is lacking of them. Also the dramatic decline of phosvitin phosphorylation caused by its previous extensive dephosphorylation has been attributed to the disappearance of polyphosphorylserine blocks [15]. Probably the optimizing effect of Ser$(P)_n$ runs is due to the input of large acidic areas on the substrate molecules which makes easier its binding to the enzyme, since acetylated phosvitin, though being no more able to be phosphorylated, displays an even increased affinity for the enzyme, as shown by the K_i value of its competitive inhibition toward phosvitin phosphorylation resulting significantly lower than the K_m for phosvitin itself. By the way this would suggest that amino groups, though critical for the overall protein kinase reaction, are not required for the substrate binding which is apparently even improved by the elimination of their positive charge. Possibly the same binding effect due to several phosphorylserine residues in a row can be induced also by large amounts of aspartic and glutamic acids, as found to be present in cytosol phosphopeptides [5] which are actively phosphorylated by this enzyme though they are probably lacking of Ser$(P)_n$ blocks (unpublished data).

In conclusion we may try to suggest that the peptidic unit still able to undergo phosphorylation by rat liver cytosol phosvitin kinase is made up by one or more phosphorylserine residues having in their close proximity a lysine residue, critical for the transphosphorylation reaction, but not for the binding to the enzyme which is probably due to other residues acidic in nature.

References

1 Langan, T.A. (1967) Regulation of Nucleic Acid and Protein Biosynthesis, BBA Library, Vol. 10, pp. 233—242, Elsevier, Amsterdam
2 Rask, L., Walinder, O., Zetterqvist, O. and Engström, L. (1970) Biochim. Biophys. Acta 221, 107—113
3 Kleinsmith, L.J. and Allfrey, V.G. (1969) Biochim. Biophys. Acta 175, 123—135
4 Pinna, L.A., Clari, G. and Moret, V. (1971) Biochim. Biophys. Acta 236, 270—278
5 Pinna, L.A., Donella, A. and Moret, V. (1973) FEBS Lett. 37, 183—187
6 Allerton, S.E. and Perlmann, G.E. (1965) J. Biol. Chem. 240, 3892—3898
7 Taborsky, G. (1968) J. Biol. Chem. 243, 6014—6020
8 Shainkin, R. and Perlmann, G.E. (1971) J. Biol. Chem. 246, 2278—2284
9 Clark, R.C. (1973) Biochim. Biophys. Acta 310, 174—187
10 Walsh, D.A. and Krebs, E.G. (1973) The Enzymes (Boyer, P.D., ed.), Vol. 8, pp. 580—581, Academic Press, New York
11 Williams, J. and Sanger, F. (1959) Biochim. Biophys. Acta 33, 294—296
12 Heald, P.J. (1961) Biochem. J. 80, 510—514
13 Sperti, S., Lorini, M., Pinna, L.A., Moret, V. and Siliprandi, N. (1964) Biochim. Biophys. Acta 82, 476—480
14 Rabinowitz, M. and Lipmann, F. (1960) J. Biol. Chem. 235, 1043—1050
15 Mano, Y. and Lipmann, F. (1966) J. Biol. Chem. 241, 3834—3837
16 Mecham, D.K. and Olcott, H.S. (1949) J. Am. Chem. Soc. 71, 3670—3679
17 Cohen, J.A., Oosterbaan, R.A. and Berends, F. (1967) Methods in Enzymology (Hirs, C.H.W., ed.), Vol. XI, p. 699, Academic Press, New York
18 Riordan, J.F. and Vallee, B.L. (1967) Methods in Enzymology (Hirs, C.H.W., ed.), Vol. XI, pp. 565—566, Academic Press, New York
19 Baggio, B., Pinna, L.A., Moret, V. and Siliprandi, N. (1970) Biochim. Biophys. Acta 212, 515—517

20 Kish, V.M. and Kleinsmith, L.J. (1974) J. Biol. Chem. 249, 750—760
21 Lindberg, O. and Ernster, L. (1956) Methods in Biochem. Analysis (Glick, D., ed.), Vol. 3, p. 8, Interscience, New York
22 Martin, J.B. and Doty, O.M. (1949) Anal. Chem. 21, 965—967
23 Wagner, H. (1960) Fette Seifen 62, 1119—1123
24 Hnilica, L.S. (1972) The Structure and Biological Functions of Histones, pp. 84—88, C.R.C. Press, Cleveland
25 Ando, T. and Watanabe, S. (1969) Int. J. Protein Res. 1, 221
26 Nohara, H., Takahashi, T. and Ogata, K. (1966) Biochim. Biophys. Acta 127, 282—284
27 Taborsky, G. and Connelly Allende, C. (1962) Biochemistry 1, 406—411
28 Fruchter, R.G. and Crestfield, A.M. (1965) J. Biol. Chem. 240, 3868—3874
29 Balls, A.K. and Wood, H.N. (1956) J. Biol. Chem. 219, 245—256