PHOSPHORYLATION OF α_{s2}-CASEIN BY TWO RAT LIVER 'CASEIN KINASES'

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1. Introduction

Phosphorylation of whole casein by two rat liver protein kinases (casein kinases TS and S) is a discriminating process involving some casein subfractions in preference to others [1,2]. In particular practically all the ^{32}P incorporated into whole casein in the presence of casein kinase S and TS is accounted for by a minor casein fraction identified as α_{s2}-casein [3]. The recent characterization of α_{s2}-casein as a single defined protein [4] and the elucidation of its primary structure [5] prompted us to study the phosphorylation of purified α_{s2}-casein by the two rat liver protein kinases in order to define more precisely the substrate specificity and the structural requirements of these enzymes. The results, while confirming the suitability of α_{s2}-casein as substrate for both the protein kinases tested, also showed that different sites are phosphorylated by the two enzymes. Moreover the knowledge of the amino acid sequences around the identified phosphorylation sites allows some interesting comparisons between the structural requirements of our 'casein kinases' and those of other known protein kinases [6–9].

2. Experimental

'Casein kinases' TS and S were prepared from rat liver cytosol by combined Sepharose 6B and Phosphocellulose column chromatographies, as in [1].

α_{s2}-Casein was prepared according to [4] from the milk of individual cows of either French-'Frisonne' or Bavarian race. Quite similar results were obtained in the two cases. Samples of purified casein fractions were kindly supplied by the Laboratoire de Recherches sur les Protéines, INRA, Jouy-en-Josas.

Phosphorylation of casein fractions was performed at 37°C by incubating them, at final conc. 5 mg/ml (unless differently indicated), for 15–30 min in a medium containing: 100 mM Tris–HCl buffer, pH 7.5; 12 mM $MgCl_2$; 50 μM [γ-^{32}P]ATP, with a spec. radioact. ~25 μCi/μmol and either casein kinase S (5–10 μg) or casein kinase TS (1–2 μg). The reaction was stopped by addition of trichloroacetic acid to 10% (w:v) final concentration. The phosphoprotein was recovered by centrifugation, washed 4 times by resuspending with 5 ml 10% trichloroacetic acid and dissolved in the minimum volume of 0.1 M NH_4HCO_3, raising the pH, if necessary, to 7.5 by small additions of NaOH. Aliquots of solubilized phosphoprotein were than either counted in a liquid scintillator (Packard model 385) or submitted to further procedures (gel electrophoresis, digestion with CNBr).

α_{s2}-Casein, 30% dephosphorylated, was prepared by 30 min incubation with potato acid phosphatase (Boehringer Grade II) under the conditions in [5]. The dephosphorylation was stopped by adding trichloroacetic acid to final conc. 5% and the mixture centrifuged. The resultant pellet was dissolved with 0.5 M Tris–HCl, pH 7.5, and dialyzed against water.

Polyacrylamide-gel electrophoresis, 7.5%, of α_{s2}-[^{32}P]casein were run at pH 8.9, essentially according to [10] with further addition of 5 M urea and 0.5% mercaptoethanol. After staining with Coomassie brilliant blue the gel column was sliced into 2 mm sections which were counted in a scintillator. Parallel standards were constantly run with α_{s1}- β- and κ-caseins, either separated or together.

CNBr digestion of α_{s2}-[^{32}P]casein and the separation of peptide CN$_5$, insoluble at pH 1.9, from peptides CN$_1$ to CN$_4$ were performed according to [4]. The mixture of the peptides CN$_1$–CN$_4$ was furtherly resolved into its 4 components by gel filtration through a 1.8 × 69 cm Sephadex G-50 column equilibrated with 15% formic acid (v:v) and operated with a 12 ml/h flow rate. Fractions, 2.7 ml, were collected. The column was pre-calibrated with the following peptides: salmine sulfate (mol. wt ~5500); insulin A chain (mol. wt ~2500); and oxidized glutathione (mol. wt 620).

The CN$_5$ peptide, insoluble at pH 1.9, was submitted to tryptic digestion in NH$_4$HCO$_3$ according to [11]. After digestion the sample was made 15% with formic acid and applied to the above described Sephadex G-50 column.

The isolation of radioactive (Ser-P)$_n$ clusters from CNBr and tryptic phosphopeptides was performed after 12 N HCl hydrolysis by paper electrophoresis at pH 1.5, according to [12]. In some experiments the radioactive peptides moving faster than Ser-^{32}P toward the anode and containing the (Ser-P)$_n$ blocks were eluted from the paper and further characterized for their size (by gel filtration through a calibrated 1.8 × 70 cm Sephadex G-25 column equilibrated with 15% formic acid), amino acid composition and labelled phospho amino acid content.

Ser-^{32}P and Thr-^{32}P were isolated from labeled proteins and peptides after 6 N HCl hydrolysis (4 h at 105°C) by pH 1.9 paper electrophoresis as in [13].

Amino acid analyses were carried out in a Geol JLC-6AH apparatus after 6 N HCl hydrolysis under N$_2$ at 105°C for 24 h.

High-voltage electrophoreses of tryptic peptides were run on Whatman 3 MM paper, at pH 4.7 (pyridine/acetic acid/water, 20:20:960) for 3 h at 200 V/cm.

Phosphate was determined after HClO$_4$ digestion according to [14].

3. Results

3.1. Enzymatic phosphorylation of native α_{s2}-casein

As shown in table 1, α_{s2}-casein can be labeled by both casein kinases TS and S, in the presence of [γ-^{32}P]ATP and Mg^{2+}, much more actively than α_{s1}-casein. Ca^{2+} cannot replace Mg^{2+} as activator of the enzymes. Only serine residues are phosphorylated by casein kinase S, while both Thr-^{32}P and Ser-^{32}P could be isolated from α_{s2}-casein labeled by casein kinase TS. The demonstration that the phosphorylation by both kinases actually involves α_{s2}-casein rather than other contaminating casein fractions was provided by submitting the α_{s2}-[^{32}P]casein preparations to polyacrylamide gel electrophoresis in 5 M urea: as shown in fig.1 the peak of radioactivity exactly overlaps the α_{s2}-casein band, which, under such conditions, is quite well separated from the other casein components.

3.2. Localization of the phosphorylable residues within α_{s2}-casein primary structure

By BrCN treatment of α_{s2}-casein 5 peptides are

Table 1
Phosphorylation rates of α_{s1}- and α_{s2}-caseins by rat liver casein kinases TS and S

Casein substrate	^{32}P incorporation (cpm) with		
	Casein kinase TS		Casein kinase S
α_{s1}	9500		5610
α_{s2}	90 071	41% Thr-^{32}P 59% Ser-^{32}P	27 340 (100% Ser-^{32}P)
α_{s2}, Mg^{2+} omitted	1120		890
α_{s2}, Mg^{2+} omitted + 10 mM Ca^{2+}	1280		950

Incubation time 15 min, in the presence of 0.5% mercaptoethanol. Concentration of casein fractions was 0.5 mg/ml
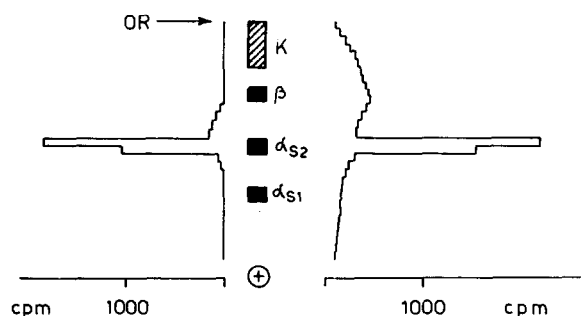
Fig.1. Polyacrylamide gel electrophoresis of α_{s_2}-[^{32}P]casein (0.5 mg) labelled by either casein kinase S (right) or TS (left).

obtained [4] : a large peptide CN_5, insoluble at pH 1.9, and peptides CN_1–CN_4, soluble at pH 1.9, which can be easily separated by Sephadex G-50 gel filtration. By applying such a procedure to α_{s_2}-[^{32}P]-casein labeled by either casein kinase S or TS it was consistently found that only peptides CN_2 and CN_5 were labelled in both instances, while the remaining peptides were unlabelled. Moreover the relative labeling of peptides CN_2 and CN_5 is sharply different depending on the phosphorylating kinase: as shown in fig.2 the peptide CN_5 accounts for all the Thr-^{32}P and most of the Ser-^{32}P residues phosphorylated by casein kinase TS, while about 80% of the radioactivity incorporated by casein kinase S is recovered in the peptide CN_2, corresponding to the sequence 5–26 and containing 5 serine residues, namely the (Ser-P)$_3$ block 8–10, an isolated Ser-P at position 16 and a
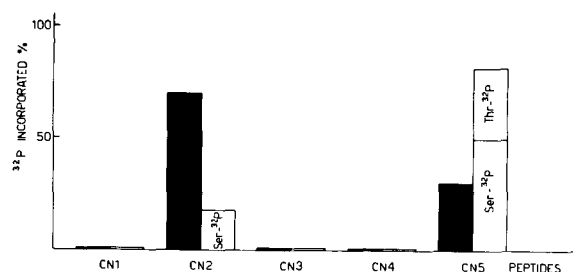

Fig.2. Distribution of the radioactivity among the CNBr peptides obtainable from α_{s_2}-[^{32}P]casein labelled by either casein kinase S or TS. The nomenclature of peptides is the same as in [4]. Aliquots of the peptides labeled by casein kinase TS were analyzed for their Ser-^{32}P and Thr-^{32}P content as in section 2.

non-phosphorylated Ser residue at position 13. In order to determine which of these residues represent the phosphorylation site(s) of casein kinase S, acid hydrolysis with 12 N HCl, which proved successful to isolate (Ser-P)$_n$ blocks from casein [12], was applied to labelled CN_2 peptide. After 24 h hydrolysis most of the radioactivity incorporated into CN_2 by kinase S was recovered in very acidic and extremely phosphorylated peptides whose size (evaluated by Sephadex G-25 gel filtration), electrophoretic mobility and amino acid composition are consistent with their identification with the sequences 7(8)–13(14) including both the (Ser-P)$_3$ block and isolated Ser$_{13}$ but excluding the isolated Ser-P$_{16}$ residue. By further acid hydrolysis however (12–36 h) the radioactivity was gradually converted into free Ser-^{32}P, while it proved impossible to detect labeled (Ser-P)$_3$ and (Ser-P)$_2$ blocks. Such a result, considering the remarkable resistence of (Ser-P)$_2$ to acid hydrolysis [12] strongly suggests that the Ser residue phosphorylated by casein kinase S in the sequence 8–16 is Ser$_{13}$ rather than one of the clustered Ser-P. By the same procedure the ^{32}P-labelled CN_2 peptide derived from α_{s_2}-casein labeled by casein kinase TS, on the contrary, gave rise to detectable amounts of radioactive (Ser-P)$_2$ and probably also (Ser-P)$_3$ suggesting that the (Ser-P)$_3$ is the substrate for the reaction catalyzed by casein kinase TS.

The large peptide CN_5, preferentially labeled by the kinase TS and consisting of 115 residues containing 10 serines and 7 threonines susceptible to phosphorylation, was digested further with trypsin and the trypsin peptides were separated by Sephadex G-50 gel filtration in order to localize the labeled Ser and Thr residues. As shown in fig.3a, when the labelling enzyme is the kinase TS two radioactive peaks are obtained corresponding to peptides of mol. wt $\sim$3500 and 1500, respectively, and accounting together for all the phosphate present in the native peptide CN_5. This result together with their high anodic mobility, at pH 4.7, and their amino acid composition after electrophoretic purification is consistent with their identification as the sequences 46–70 and 126–136(137) according to the primary structure established in [5]. Moreover the additional finding that only Ser-^{32}P residues are present in the larger peptide and only Thr-^{32}P in the smaller one confine the phosphorylable sites to the sequence Ser$_{53}$–Ser$_{61}$
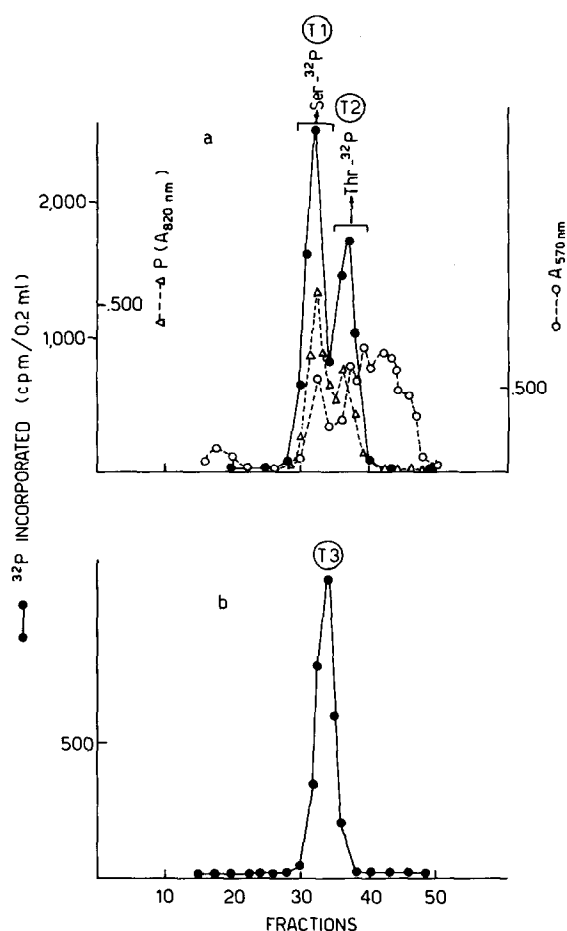
Fig.3. Sephadex G-50 gel chromatography of the tryptic digests of the ^{32}P-labelled CN_5 peptides from α_{s_2}-casein labelled by either casein kinase TS (upper figure) or S (lower figure). (•—•) ^{32}P incorporated (cpm); (o- - - -o) ninhydrin reaction (A_{570} nm); (X - - - -X) endogenous P (A_{820}). The ninhydrin and phosphate profiles of the lower figure were exactly superimposable to those of the upper figure and are not reported. The radioactivity of T_1 and T_2, collected as indicated, was accounted for by only Ser-^{32}P and Thr-^{32}P, respectively.

of the former and to the unique Thr residue, namely Thr_{130} of the latter. Indeed upon 12 N HCl hydrolysis of ^{32}P-labelled T_2 peptide, besides some free Thr-^{32}P, only very acidic di- and tri-peptides could be isolated by paper electrophoresis containing both unlabelled Ser-P and labelled Thr-^{32}P: from the primary structure of CN_5 peptide Thr_{130} is adjacent to two phosphorylated serine residues.

By applying the same procedure to the Ser-^{32}P containing peptide T_1 most of the radioactivity was again present in small peptides more acidic than Ser-P itself displaying, at pH 1.5, the electrophoretic mobility typical for $(Ser-P)_2$, $(Ser-P)_3$ and $(Ser-P)_n$—Glu blocks [12], so indicating that the Ser residue(s) phosphorylated by casein kinase TS in peptide T_1 belongs to the $(Ser-P)_3$ cluster 56—58 rather than to the isolated Ser_{53} or $(Ser-P)_{61}$ residues.

No definitive evidence about the Ser site(s) labeled by casein kinase S in peptide CN_5 and accounting for 15—30% of the whole labelling of native α_{s_2}-casein by this enzyme could be obtained. The size of the only radioactive peak obtainable by gel filtration of the tryptic digest of ^{32}P-labelled CN_5 peptide labeled by the kinase S (T_3 of fig.3b) in fact does not fit with any of the predicted Ser containing tryptic peptides from peptide CN_5, unless assuming that the de novo phosphorylation of a Ser residue close to a Lys or to an Arg residue might prevent trypsin by acting on it thus giving rise to a peptide consisting of two predicted tryptic peptides still linked together. The size of T_3, intermediate between the largest and the largest but one Ser containing predicted tryptic peptides (46—70 and 126—133, respectively) and its high endogenous phosphate content might suggest its identification with the fragment 126—141, assuming the phosphorylation of Ser_{135} close to Lys residues 136 and 137.

3.3. Enzymatic phosphorylation of partially dephosphorylated α_{s_2}-casein

Further strong experimental support for different requirements of the two kinases comes from the results of phosphorylation of 30% dephosphorylated α_{s_2}-casein. As shown in fig.4 the previous dephosphorylation promotes a several-fold increase of the casein kinase TS-dependent phosphorylation of Ser residues in both peptides CN_2 and T_1, while the phosphorylation of residue Thr_{130} becomes neglegible. The phosphorylation of both whole α_{s_2}-casein and peptides CN_2 and T_3 by casein kinase S on the contrary is unaffected by the previous dephosphorylation. These findings are consistent with the conclusion that casein kinase TS displays a specific activity toward Ser residues alse preferred by the physiological casein kinase, while the phosphorylation by casein kinase S involves ser residues not phosphorylated in
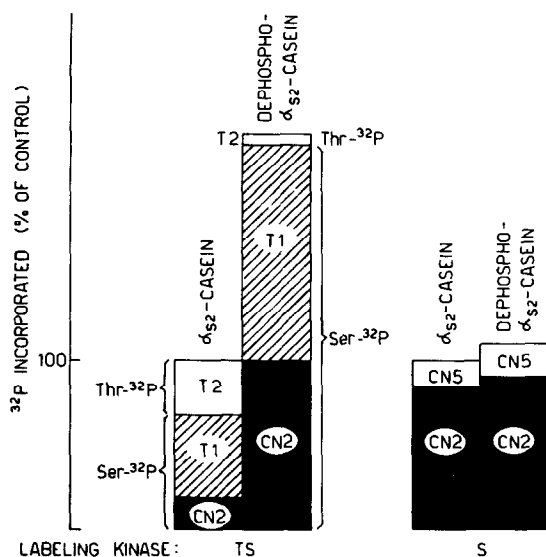
Fig.4. Phosphorylation of native and 30% dephosphorylated α_{s2}-casein by casein kinases TS and S. Peptides nomenclature is the same used in fig.2,3.

the native casein, and therefore fully available even before dephosphorylation.

4. Discussion

The results reported in the present paper provide clearcut evidence for the suggestion [2,3] that α_{s2}-casein represents a particularly efficient model substrate for two rat liver cAMP-independent protein kinases; namely so-called 'casein kinases TS and S' [1]. Indeed purified α_{s2}-casein displays with both kinases, phosphorylation rates several-fold higher than those observed with the main casein component, i.e., α_{s1}-casein. Moreover the identification of the regions and in some instances of the residues undergoing phosphorylation allows some interesting speculations about the structural requirements of these two enzymes, whose natural substrates are still unknown. In particular casein kinase TS displays a remarkable specificity for residues which are also suitable for the physiological casein kinase responsible for the in vivo phosphorylation of caseins within the mammary gland. This conclusion rests on the following findings:

1. The regions which are highly phosphorylated in native α_{s2}-casein are also those specifically labeled by casein kinase TS.
2. Thr_{130}, which can be viewed as a potential phosphorylation site for the physiological casein kinase since it fulfils the structural requirements postulated for this enzyme [5], is actively phosphorylated by our kinase TS.
3. Previous dephosphorylation of native α_{s2}-casein by acid phosphatase causes a dramatic increase in the phosphorylation rate by casein kinase TS, as expected by assuming that the dephosphorylation makes available to the kinase many new Ser sites formerly in the phosphorylated form, while depressing, through a competitive effect, that toward Thr_{130}, as experimentally observed. It should be recalled that native α_{s2}-casein molecules are characterized by a variable degree of phosphorylation [4]: probably the incomplete phosphorylation of the 8–10 and 56–58 Ser-P blocks in a few molecules, together with the availability of Thr_{130} in all molecules (see fig.5) are responsible for the phosphorylation of native α_{s2}-casein by casein kinase TS.

The structural requirements of casein kinase S on the other hand must be quite different from those of both casein kinase TS and mammary gland casein kinase since neither Thr_{130} nor the Ser residues in the 56–61 region (see fig.5) are phosphorylated either before or after enzymatic dephosphorylation, which also fails to stimulate the overall phosphorylation by this kinase. Such a conclusion is also in good agreement with the finding that ^{32}P incorporated into α_{s2}-casein by casein kinases TS and S displays different sensitivities to a cytosol casein phosphatase [2]. Indeed a large amount of ^{32}P incorporated into native α_{s2}-casein by casein kinase S is recovered in a Ser-^{32}P residue near the N-terminal, probably Ser_{13}: such a residue is not a potential site of phosphorylation by the physiological casein kinase since it lacks the critical acidic group two residues to its C-terminal, though a very acidic sequence (Ser-P_3–Glu_2) is located to its N-terminal. Interestingly a quite similar acidic sequence is also located to the N-terminal of Ser_{135}, which is indirectly suspected to represent a second phosphorylation site of α_{s2}-casein by casein kinase S.

The above conclusions are summarized in fig.5

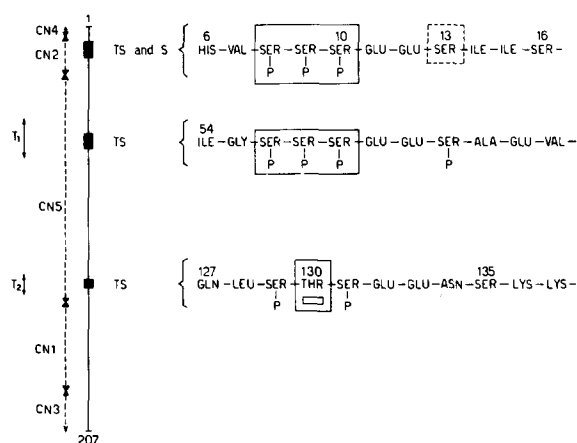Fig.5. α_{s_2}-Casein regions labeled by casein kinases TS and S and amino acid sequences around the phosphorylable sites. (—■—) Regions of the native α_{s_2}-casein primary structure with high endogenous P content [5]. The regions phosphorylated by casein kinases TS and S according to the present paper are indicated on the right of α_{s_2}-casein peptide chain. On the left are indicated the peptides obtained by CNBr digestion and the tryptic peptides (T_1 and T_2) labeled by casein kinase TS. (□) Potential site of phosphorylation for the physiological casein kinase according to [5]. Continous (□) and dotted (□) squares include the residues probably affected by casein kinases TS and S, respectively.

where the amino acid sequences in the regions of α_{s_2}-casein undergoing labeling by casein kinases TS and S are reported. The sites either phosphorylated or potentially phosphorylable by the endogenous casein kinases and those labeled by our two kinases are also indicated. The overlapping of the regions phosphorylable in vivo with those labeled by casein kinase TS confirms once more that the latter enzyme displays structural requirements similar if not identical to that reported for the mammary gland casein kinase consisting of an acidic group (Ser-P or Glu) two residues to the C terminal of the phosphorylable site [4,7]. The phosphorylation by casein kinase S is evidently dependent on different requirements, possibly consisting of an acidic region proximal to the N-terminal of the phosphorylable site. At present however such a latter suggestion is just a working hypothesis requiring further experimental evidence.

Casein kinase S, as well as casein kinase TS apparently do not display the site specificity pro-

posed for protein kinases active on histones and on several catalytic proteins, requiring two basic residues in the proximity of the N-terminal of the phosphorylated residue [9]. None of the residues phosphorylated in α_{s_2}-casein in fact fulfils such a requirement, while Ser_{168} and Thr_{138}, both having near to their N-terminals a couple of ajacent Lys residues proved completely inactive as phosphate acceptors in the presence of both the casein kinases used in the present study.

Acknowledgements

References

[1] Meggio, F., Donella Deana, A., Pinna, L. A. and Moret, V. (1977) FEBS Lett. 75, 192–196.
[2] Pinna, L. A., Donella Deana, A. and Meggio, F. (1977) Biochem. Biophys. Res. Commun. 78, 8–15.
[3] Donella Deana, A., Meggio, F., Pinna, L. A. and Moret, V. (1978) Biochim. Biophys. Acta 524, 316–326.
[4] Brignon, G., Ribadeau-Dumas, B. and Mercier, J. C. (1976) FEBS Lett. 71, 111–116.
[5] Brignon, G., Ribadeau-Dumas, B., Mercier, J. C. and Pelissier, J. P. (1977) FEBS Lett. 76, 274–279.
[6] Mercier, J. C., Grosclaude, F. and Ribadeau-Dumas, B. (1971) Eur. J. Biochem. 23, 41–51.
[7] Mackinlay, A. G., West, D. W. and Manson, W. (1977) Eur. J. Biochem. 76, 233–243.
[8] Edlund, B., Zetterqvist, O., Ragnarsson, U. and Engstom, L. (1977) Biochem. Biophys. Res. Commun. 79, 139–144.
[9] Shenolikar, S. and Cohen, P. (1978) FEBS Lett. 86, 92–98.
[10] Maurer, H. R. (1971) in: Disc Electrophoresis, p. 44, de Gruyter, Berlin, New York.
[11] Grosclaude, F., Mercier, J. C. and Ribadeau-Dumas, B. (1970) Eur. J. Biochem. 14, 98–107.
[12] Williams, J. and Sanger, F. (1959) Biochim. Biophys. Acta 33, 294–296.
[13] Moret, V., Clari, G. and Pinna, L. A. (1975) Biochim. Biophys. Res. Commun. 62, 1011–1017.
[14] Wagner, H. (1960) Fette Seifen 62, 1119–1123.
[15] Fruchter, R. G. and Crestfield, A. M. (1965) J. Biol. Chem. 240, 3868–3874.