ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 2 (1996) 85–92

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Oxyanion formation in phosphoryl transfer catalyzed by protein kinases A and C

Jaak Järv [1]

Institute of Chemical Physics, University of Tartu, 2 Jakobi Str., EE 2400 Tartu, Estonia

Received 7 December 1995; accepted 24 April 1996

Abstract

The chemical mechanism of phosphoryl transfer from ATP to substrate hydroxyl group, catalyzed by protein kinases A and C, was analyzed in terms of the transition state structure of the rate-limiting step of the reaction. The analysis was based on quantitative structure–activity relationships, obtained by applying the kinetic data on phosphorylation of series of amino alcohols coupled to active-site directed peptides and published by Kwon et al. (*J. Biol. Chem.*, 269 (1994) 4839–4844) and by Kwon et al. (*J. Biol. Chem.*, 168 (1993) 10713–10716). The analysis revealed the significant role of the inductive effect in these reactions, characterized by the ρ^* values 7.6 and 4.8 for protein kinases A and C, respectively. The removal of the proton from the phosphorylatable hydroxyl group and the formation of oxyanion on the rate-limiting step of the catalysis were suggested on the basis of the intensity factors ρ^*.

Keywords: Oxyanion; Phosphoryl transfer; Protein kinases; Structure–activity relationships

1. Introduction

Modulation of cellular processes through regulatory phosphorylation is catalyzed by protein kinases transferring the γ-phosphate of ATP to serine, threonine or tyrosine residues of the acceptor protein or peptide [1]. These enzymes reveal different substrate specificity, recognizing the structure of the phosphorylatable amino acid as well as the amino acid sequence around this reaction center [2]. On the other hand, the mechanism of the bond-breaking step of the phosphorylation reaction shows rather similar features across the family of protein kinases, as the catalytic domains of these enzymes contain a number of conserved sequences.

The direct in-line phosphoryl transfer mechanism of serine phosphorylation has been suggested on the basis of the three-dimensional structure of the complex of cAMP-dependent protein kinase (protein kinase A) with peptide and ATP [3]. This mechanism is supported by the fact that the inversion of configuration at the phosphorus atom has been observed in the phosphoryl group transfer from ATP to serine peptide [4]. The viscosity-dependent steady-state

[1] Fax: (+372-7)465 247 Phone: (+372-7)465 246; E-mail: JJ@CHEM.UT.EE

PII S1381-1177(96)00012-4

kinetic data have shown that the covalent step of the catalysis may be a fast process followed by the rate-limiting release of the second product, ADP [5].

On the basis of crystallographic data a magnesium ion complex has been identified in the catalytic pocket of protein kinase A. Therefore the electrophilicity of the γ-phosphorus atom of the ATP molecule seems to be increased through the mechanism of general acid catalysis [3]. On the other hand, participation of a basic group in the catalytic act has been proposed proceeding from the pH dependences of the steady-state kinetic parameters [6]. This basic group should assist by removing the hydroxyl proton from the phosphorylatable serine concomitant with the nucleophilic attack of this group on the γ-phosphate of ATP. Crystallographic data have shown that this catalytic group may be the Asp 166 in the protein kinase A active center [3]. The participation of this residue in catalysis has been shown by replacement of the Asp with Ala in the yeast enzyme, which reduced the V value about 300-fold without significantly affecting the K_m for peptide and ATP [7].

Owing to the non-chemical nature of the rate-limiting step of the overall enzyme reaction, the pK_a value of 6.1 for this putative basic catalytic group has been revealed in the case of the second-order kinetic constants $V/K_{m,peptide}$, but has not been observed in the pH dependences of maximal velocity [6]. On the other hand, Adams and Taylor [8] have found that the appearance of this functional group in the pH dependences of $V/K_{m,peptide}$ required the presence of an arginine residue at the -2 position of the minimum consensus sequence $R_{-3}R_{-2}X_{-1}SX_1$ of the protein kinase A substrates. Therefore they concluded that recognition of the arginine residue by the enzyme also needs ionization of an enzyme basic group. Thus, the participation of a basic group in the catalytic mechanism of the phosphoryl transfer reaction is still by no means completely understood. Moreover, if the basic group really participates in the catalysis, the pH dependences and crystallographic data alone cannot clearly show whether the group actually removes the proton to enhance the nucleophilicity of the substrate OH group, or whether it helps to bind the substrate in the correct orientation at the active site.

This paper presents a further analysis of these aspects of the catalytic mechanism of peptide phosphorylation, proceeding from the quantitative structure–activity relationships for a series of protein kinase substrates. The appropriate kinetic data for protein kinase A and protein kinase C have been published by Kwon and coworkers [9,10]. The results of this analysis suggested oxyanion formation on the covalent step of the phosphoryl transfer reaction catalyzed by the both protein kinases.

2. Kinetic data and substituent constants

The substrates under consideration have been prepared and studied by Kwon and coworkers [9,10]. The general structure of these compounds can be presented as follows:

$$\text{peptide–NH–X}_i\text{–OH} \qquad (1)$$

and they contain structurally diverse alcohol-bearing residues $-X_iOH$ coupled to the C-terminal part of peptides via an amide bond. The peptides attached were specially designed by Kwon and coworkers [9,10] to meet the specificity requirements of protein kinase A and protein kinase C and to provide effective phosphorylation of the alcohol group by these enzymes.

As the steady-state kinetic constants of phosphorylation of Eq. (1) have been found to be close to the appropriate kinetic data for specific peptide substrates of these protein kinases, it has been concluded that the phosphorylation of all these compounds proceeded via a comparable kinetic mechanism. On the basis of this assumption the peptide derivatives Eq. (1) had

Table 1
Second order rate constants

No.	$-X_i-OH$	Protein kinase A $\log k_{II}$	Protein kinase C $\log k_{II}$
I	$-CH(CONH_2)CH_2OH$	0.09	−1.26
II	$-(CH_2)_2OH$	−1.14	−1.53
III	$-(CH_2)_3OH$	−2.04	−2.10
IV	$-(CH_2)_4OH$	−3.66	−3.60
V	$-CH(CH_3)CH_2OH$	−0.56	−1.62
VI	$-CH(C_2H_5)CH_2OH$	−0.86	−1.74
VII	$-CH(CH_2Ph)CH_2OH$	−0.77	−2.48
VIII	$-CH(iBu)CH_2OH$	−1.06	−3.25
IX	$-CH(CH_2SCH_2Ph)CH_2OH$	0.46	−2.08
X	$-CH(CH_2OCH_2Ph)CH_2OH$	–	−1.61
XI	$-CH(CONHCH_2CH_2Ph)CH_2OH$	–	−1.24

The log k_{II} values for phosphorylation of Gly–Arg–Thr–Gly–Arg–Arg–Asn–X_i–OH by protein kinase A and Leu–Arg–Arg–Arg–Arg–Phe–X_i–OH by protein kinase C were calculated from the V and K_m values published by Kwon and coworkers [9,10].

been used to study the active site substrate specificity of protein kinases A and C [9,10].

For the following correlation analysis the second-order rate constants k_{II} (Table 1) were calculated as ratios of the steady-state kinetic parameters V and K_m published by Kwon and coworkers [9,10]. As the latter kinetic parameters have been obtained in the presence of an ATP excess, the second-order rate constants correspond to the ratio k_2/K_S, where K_S is the effectiveness of the substrate (peptide) in binding to the enzyme–MgATP complex and k_2 is the rate of the following step of the enzyme reaction. Thus, compared to V and K_m, the meaning of k_{II} is more concrete and depends less on the specific features of the kinetic mechanism or on the nature of the rate-limiting step of the overall process [11].

The reaction series for the following correlation analysis were composed proceeding from the following principles.

First, the hydroxyl group of the alcohol-bearing residues of substrates (1) was defined as the 'reaction center' and the structurally diverse part $C(O)NHX_i-$ of these molecules was defined as the 'substituent'.

Secondly, the fragments $-C(O)NHX_iOH$ containing only the primary alcohol group were selected to avoid uncertain stereoselectivity effects at the β-carbon of the amino alcohol residue.

Thirdly, only the compounds in which the structural fragment $-X_i-$ resembles the stereochemical configuration of L-serine, or has no chiral center, were used in the following analysis to avoid problems connected with stereospecificity of protein kinases.

Table 2
Substituent constants for correlation analysis

No.	$-X_i-OH$	σ^* $C(O)NHX_i$	E_s^o $C(O)NHX_i$	MR R_α	π R_α
I	$-CH(CONH_2)CH_2OH$	0.53	−1.13	9.81	−1.49
II	$-(CH_2)_2OH$	0.26	−0.6	1.03	0
III	$-(CH_2)_3OH$	0.10	−0.6	1.03	0
IV	$-(CH_2)_4OH$	0.04	−0.6	1.03	0
V	$-CH(CH_3)CH_2OH$	0.26	−1.13	5.65	0.56
VI	$-CH(C_2H_5)CH_2OH$	0.26	−1.13	10.3	1.02
VII	$-CH(CH_2Ph)CH_2OH$	0.30	−1.13	25.36	2.01
VIII	$-CH(iBu)CH_2OH$	0.26	−1.13	19.61	1.80
IX	$-CH(CH_2SCH_2Ph)CH_2OH$	0.37	−1.13	43.59	3.1
X	$-CH(CH_2OCH_2Ph)CH_2OH$	0.38	−1.13	36.98	0.72
XI	$-CH(CONHCH_2CH_2Ph)CH_2OH$	0.53	−1.13	42.37	0.79

The σ^* and E_s^o values for the $C(O)NHX_i-$ groups were obtained from Palm et al. [12], the MR and π values for the α-substituents R in $C(O)NHCH(R_\alpha)CH_2OH$ were taken from [11].

The conventional set of substituent constants was used in the structure–activity analysis (Table 2). This set contains inductivity constants for aliphatic substituents (σ^*) [12], Taft steric constants (E_s^o) [12,13], hydrophobicity parameters (π) [12] and molecular refractivity constants (MR) [12], characterizing the 'bulkiness' of substituents.

The constants σ^* and E_s^o were used to take into account the changes in the 'intrinsic' reactivity of substrates and, therefore, these parameters were calculated for the whole structural fragment $-C(O)NHX_i-$, while the alcohol group attached was regarded as the 'reaction center'. The inductivity constants for these fragments were calculated as additive values on the basis of the standard σ^* constants listed in [12]. The steric constants were used following the 'isostericity' principle [13].

The parameters of hydrophobicity (π) and 'bulkiness' (MR) were used to analyze whether the variable part of substrates interacts with the enzyme active center. Therefore, these effects were quantified only for substituents in the α-position of the alcohol-bearing fragment. The appropriate substituent constants were calculated as described by Hansch and Leo [12]. As there was significant mutual correlation between the π and MR values, only one of these parameters was used in each correlation.

3. Quantitative structure–activity relationships

The correlation of $\log k_{II}$ values for protein kinases A and C (Table 1) with different combinations of substituent constants σ^*, E_s^o, π or MR (Table 2) revealed that the steric effect was not a significant structural factor in the present reaction series. This can be explained by the fact that variation in substrate structure occurs at some distance from the reaction center, where the steric influence levels off to some constant value. On the other hand, this preliminary analysis also demonstrated that the inductive effect was the most significant structural factor for both enzymes. Bulkiness or hydrophobicity of the substituents in the α-position of the amino alcohol fragment of the peptide derivatives (I), $-C(O)NH-CH(R_\alpha)CH_2OH$, had a somewhat stronger influence in the case of protein kinase A. For a more precise analysis of these factors the following two-parameter correlation equations were used:

$$\log k_{II}(X_i) = \log k_{II}^o + \rho^*\sigma^* + \varphi\pi \quad (2)$$

or

$$\log k_{II}(X_i) = \log k_{II}^o + \rho^*\sigma^* + \psi(MR) \quad (3)$$

where ρ^* is the intensity factor of the inductive effect and φ and ψ describe the intensities of the hydrophobic effect and the substituent bulkiness.

In the case of protein kinase A these combinations of structural factors yielded correlations of a rather similar quality:

$\log k_{II}^o = -3.3 \pm 0.4$, $\rho^* = 7.6 \pm 1.2$, $\varphi = 0.3 \pm 0.1$, $r = 0.941$, $s = 0.472$, $F = 23$ for Eq. (2) and

$\log k_{II}^o = -3.1 \pm 0.4$, $\rho^* = 6.5 \pm 1.6$, $\psi = 0.022 \pm 0.015$, $r = 0.918$, $s = 0.554$, $F = 16$ for Eq. (3).

In this and the following correlations r is the correlation coefficient, s is the standard error of estimation and F is the ratio of variance between calculated and observed values. The 95% confidence intervals of the parameters are given.

It can be seen that the correlation involving the π constants is the preferred one. Omitting π and MR from the analysis, a more simple equation can be used:

$$\log k_{II}(X_i) = \log k_{II}^o + \rho^*\sigma^* \quad (4)$$

This equation yielded a somewhat less significant correlation: $\log k_{II}^o = -3.1 \pm 0.4$, $\rho^* = 7.6 \pm 1.5$, $r = 0.888$, $s = 0.596$, $F = 26$, while there was no meaningful relationship between $\log k_{II}$ and π or MR alone.

In the case of protein kinase C the Eqs. (2) and (3) led to the following results:

$\log k_{II}^{o} = -3.0 \pm 0.4$, $\rho^* = 3.5 \pm 1.2$, $\varphi = -0.18 \pm 0.15$, $r = 0.745$, $s = 0.581$, $F = 5$, and

$\log k_{II}^{o} = -3.2 \pm 0.4$, $\rho^* = 4.8 \pm 1.6$, $\psi = -0.018 \pm 0.014$, $r = 0.751$, $s = 0.574$, $F = 5$, respectively.

It can be seen that a slight preference should be given to the latter results. The correlation with inductive effect alone gave:

$\log k_{II}^{o} = -3.1 \pm 0.4$, $\rho^* = 3.5 \pm 1.2$, $r = 0.686$, $s = 0.597$, $F = 8$.

The correlation of the $\log k_{II}$ values with π and MR alone did not yield any meaningful relationships ($r < 0.3$).

4. Catalytic site specificity of protein kinases A and C

The second-order rate constants are complex parameters describing the binding (K_S) as well as the following catalytic step (k_2). Therefore it was not surprising that the inductive effect was revealed in the reactions of both enzymes. As the ρ^* constants 7.6 and 4.8 for protein kinases A and C, respectively, were determined with sufficient reliability and were almost independent of the particular form of the correlation equation, the inductive effect can be considered to be a dominating specificity factor for both enzymes.

A clear effect of hydrophobicity of substituents at the α-position of the peptide-coupled amino alcohol residue, characterized by $\varphi = 0.3 \pm 0.1$, was observed in the case of protein kinase A. It is interesting that for the same enzyme two hydrophobic binding sites, characterized by the φ constants 1.3 and 0.4, were identified for the amino acids, located before and after the phosphorylatable serine residue [14]. The latter φ value is in agreement with the intensity factor for hydrophobic interaction calculated above. On the other hand, the manifestation of hydrophobicity so close to the catalytic center seems to reflect the hydrophobic nature of this site in general rather than the presence of some particular hydrophobic binding locus.

In contrast to this situation, the role of the α-substituents in the amino alcohol part of substrates (1) cannot be clearly identified for protein kinase C owing to remarkable errors in ψ and φ. Thus, the active site specificity of this enzyme seems to be somewhat different from that of protein kinase A. The latter conclusion is in agreement with the fact that even the D-serine in peptide substrates can be phosphorylated by protein kinase C [15].

The present analysis was intentionally restricted to problems of the phosphorylation of primary alcohol groups having a structure resembling that of the phosphorylatable serine residue. However, both protein kinases also phosphorylate the secondary alcohol group of threonine, where the branching of the substituent occurs in the immediate vicinity of the reaction center. Therefore the phosphorylation rate of the secondary alcohol groups may be expected to depend also on the steric influence quantified by means of the Taft E_s^o constants. The practical analysis of this interesting situation, however, has so far been hampered by the absence of experimental data which would make possible the separation of these effects from stereoselectivity phenomena related to recognition of substrate molecule by the enzyme active center.

As emphasized by Kwon et al. [10], direct comparison of the kinetic constants obtained for protein kinases A and C is not a meaningful procedure, since different peptides were used as carriers of the phosphorylatable alcohol groups. Therefore they discussed only those trends which have been observed within each substrate series for the same enzyme. This limitation can be removed by using quantitative structure–activity relationships and by comparing the appropriate intensity factors for different enzymes. Owing to the limited structural diversity of substrates, the present analysis embraced only two specificity factors, among which the inductive effect has significant meaning.

5. Mechanism of the phosphoryl transfer reaction

The manifestation of inductive effect was in good agreement with the proposed nucleophilic mechanism of the phosphoryl transfer from ATP to the hydroxyl group of substrate. Details of this mechanism can be analyzed in terms of the transition state structure on the basis of the relationship between the ρ^* values and the inductivity constants of the *reaction center* in the initial and transition states, σ_Y^* and $\sigma_{Y'}^*$, respectively [16]:

$$\rho^* = \alpha^*(\sigma_Y^* - \sigma_{Y'}^*) \quad (5)$$

where α^* is a universal constant of the inductive interaction of aliphatic substituents presented on the scale of the log k values.

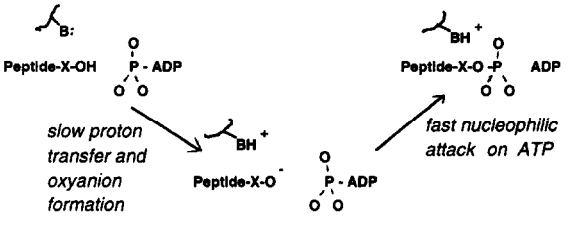
The validity of this equation was analyzed by Koppel et al. [17] in different reactions. For chemical equilibria such as dissociation of carboxylic acids, alcohols, alkylammonium ions and alkylthiols the structure of the reaction center can be clearly defined in the final state. This analysis has yielded the value of the universal inductivity constant as 2.72 ± 0.15 kcal/mol corresponding to $\alpha^* = 2.0$ in Eq. (5) [17]. The same relationship was also valid in the case of the alkaline hydrolysis of carboxylic esters and amides, where the transition state structures were estimated on the basis of the ideas concerning the mechanism of these reactions [17].

The inductivity constants σ_Y^* and $\sigma_{Y'}^*$ correspond to the common σ^* scale and can be taken from the appropriate tables or calculated additively in the case of more complex structures. On the other hand, Eq. (5) can be used for calculation of unknown $\sigma_{Y'}^*$ values from ρ^*, α^* and σ_Y^* for the purpose of analyzing the structure of the transition state of the reaction. This approach has been used in the case of the cholinesterase-catalyzed reactions [18].

In the substrates depicted in Eq. (1) the hydroxyl group of the amino alcohol part of peptide derivatives was defined as the reaction center. This means that the σ^* value 1.41 [12] for this group can be used for σ_Y^* in Eq. (5), yielding the $\sigma_{Y'}^*$ values -2.4 and -1.0 for protein kinase A and C, respectively. The negative value of the latter constants excluded the transition state structures containing the O–P bond (or at least a partially formed O–P bond), as in this case the reaction center should behave as a typical electronegative substituent characterized by $\sigma_{Y'}^* > 0$ [11].

The negative $\sigma_{Y'}^*$ values point to the electropositive nature of the reaction center in the transition state. This can be related to the removal of the proton from the OH group, as the σ^* value for oxyanion O^- remains between 0 in water [17] and -2.4 in the gas phase [19]. In the case of protein kinase A the value $\sigma_{Y'}^* = -2.4$ coincides with the latter constant, suggesting complete proton removal from the alcohol group and formation of an 'unsolvated' oxyanion on the rate limiting step of the phosphoryl transfer reaction. This process seems to occur in an aprotonic reaction medium like dimethylsulphoxide or dimethylformamide, which, similarly to that in the gas phase lacks the proton–donor ability. Thus the transition state of the enzyme reaction should be isolated from water as well as from other proton donors able to solvate this oxyanion.

On the other hand, the process of proton removal from the hydroxyl group implies participation of a basic catalytic residue (B in Scheme 1). Most probably the acidity (basicity) of this group is strongly affected by the apolar nature of the interior of the catalytic center. The oxyanion formed under such conditions should



Scheme 1.

be a highly nucleophilic reagent providing rapid phosphoryl transfer from ATP to the substrate.

The same principal mechanism seems to be valid for protein kinase C. However, in this case the transition state of the phosphorylation reaction was characterized by $\sigma_{Y'}^{*} = -1.0$, pointing either to incomplete proton removal from the OH group, or to the possibility of some weak electrophilic solvation of the oxyanion. The phenomenon that electrophilic solvation of anionic substituents (O^-, COO^-, S^-) makes them more electronegative and results in increase in their σ^* values has been discussed by Koppel et al. [20].

The results described above agree, in general, with the reaction mechanism which has been proposed by Yoon and Cook [6]. This mechanism suggested participation of a basic amino acid residue in catalysis and the present results specify the role of this putative catalytic group. Figuratively this mechanism is presented in Scheme 1 where the basic group is denoted by B. Also it is assumed that the intermediate complex structure involving the oxyanion resembles that of the transition state of the rate-limiting step of proton removal from the hydroxyl group.

The proximity in location of the substrate OH group and the Asp 166 in protein kinase A active center has been taken as an implication of the key role of this carboxylic residue in the catalysis [3]. This means that the same residue in anionic form can assist the reaction. It can be predicted that basicity of the appropriate carboxylate residue can well be increased by its aprotic surroundings in the catalytic center. This, in turn, should facilitate the formation of a highly nucleophilic oxyanion from the nucleophilic OH group of substrate. On the other hand, the same specific microenvironment in the reaction center can also explain the possibility that the pK_a of this basic group was not manifested in the pH dependences of the kinetic parameters measured within the conventional pH interval [8].

It can be mentioned that the formation of a 'naked' anion in the enzyme active centre has been proposed in the case of acetylcholinesterase and butyrylcholinesterase reactions with organophosphorus thioesters, acting as irreversible inhibitors of these enzymes. However, in these cases the unsolvated anion corresponded to the leaving group formed from the inhibitor molecule due to the complete cleavage of the phosphorus–sulphur bond in the transition state of the enzyme phosphorylation step [21,22].

Acknowledgements

This work was supported by the International Science Foundation Grants LCK 000 and LCI 100 and by the Estonian Science Foundation Grant ETF 49.

References

[1] E.G. Krebs, Enzymes, 17 (1986) 3.
[2] B.E. Kemp and R.B. Pearson, Trends Biochem. Sci., 15 (1990) 342.
[3] Madhusudan, E.A. Trafny, N.-H. Xuong, J.A. Adams, L.F.T. Eyck, S.S. Taylor and J.M. Sowadski, Protein Sci., 3 (1994) 176.
[4] M. Ho, N.H. Bramson, D.E. Hansen, J.R. Knowles and E.T. Kaiser, J. Am. Chem. Soc., 110 (1988) 2680.
[5] J.A. Adams and S.S. Taylor, Biochemistry, 31 (1992) 8516.
[6] M.-Y. Yoon and P.F. Cook, Biochemistry, 26 (1987) 4118.
[7] C.S. Gibbs and M.J. Zoller, J. Biol. Chem., 266 (1991) 8923.
[8] J.A. Adams and S.S. Taylor, J. Biol. Chem., 268 (1993) 7747.
[9] Y.-G. Kwon, M. Mendelow, J. Srinivasan, T.R. Lee, S. Pluskey, A. Salerno and D.S. Lawrence, J. Biol. Chem., 268 (1993) 10713.
[10] Y.-G. Kwon, M. Mendelow and D.S. Lawrence, J. Biol. Chem., 269 (1994) 4839.
[11] W.W. Cleland, Biochemistry, 14 (1975) 3220.
[12] C. Hansch and A. Leo, Substituent Constants for Correlation Analysis in Chemistry and Biology, John Wiley & Sons, New York, 1979.
[13] I.V. Talvik and V.A. Palm, Org. React. (Tartu), 8 (1971) 445.
[14] J. Järv and U. Ragnarsson, Bioorg. Chem., 19 (1991) 77.
[15] M. Eller, J. Järv, R. Toomik, U. Ragnarsson, P. Ekman and L. Engström, J. Biochem., 114 (1993) 177.
[16] V.A. Palm (Ed.), Fundamentals of Quantitative Theory of Organic Reactions. Khimya, Leningrad, 1977, p. 151–157.

[17] I. Koppel, M. Karelson and V. Palm, Org. React. (Tartu), 10 (1973) 497.
[18] J.L. Järv and Ü.L. Langel, Bioorg. Khim., 7 (1981) 217.
[19] I. Koppel and M. Karelson, Org. React. (Tartu), 12 (1975) 985.
[20] I. Koppel, M. Karelson and V. Palm, Org. React. (Tartu), 11 (1974) 101.
[21] Ü. Langel and J. Järv, Biochim. Biophys. Acta, 525 (1978) 122.
[22] J.L. Järv and Ü.L. Langel, Bioorg. Khim., 5 (1979) 746.