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Note

Determination of p*I* values of variously methylated amino acids by isoelectric focusing

WOON KI PAIK*, JAMAL Z. FAROOQUI, TAMAL ROY and SANGDUK KIM
Fels Research Institute and Department of Biochemistry, Temple University School of Medicine, Philadelphia, PA 19140 (U.S.A.)
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Amino acids containing side-chains modified with a methyl group occur ubiquitously in nature as free or protein-bound forms[1]. Unlike acetylation or phosphorylation which stoichiometrically neutralizes or acidifies respectively, the effect of methyl substitution on the overall charge state of an amino acid is not simple. For example, in addition to hydrophobic[2] and steric effects, substitution of one of the hydrogen atoms of ammonia with a methyl group increases the basicity (decreases the pK_b value from 4.75 to 3.37)[3,4]. The effect of a second methyl substitution is less than the first (pK_b = 3.22), and when a third methyl group is introduced the pK_b value increases to 4.20. Since we have recently been successful in determining the isoelectric points, p*I*, of various compounds of small molecular weight using the isoelectric focusing (IEF) technique[5], we felt it of interest to extend this IEF technique to the determination of p*I* values of variously methylated amino acids and to examine the generality of the above theoretical consideration.

MATERIALS AND METHODS

Materials

Uniformly ^{14}C-labeled L-lysine, L-arginine and L-histidine with specific activities of more than 300 mCi/mmol were purchased from Amersham Radiochemicals (Arlington Heights, IL, U.S.A.). DL-Homocysteine was obtained from Aldrich (Milwaukee, WI, U.S.A.). Ampholine (pH 3.5–10 and 9.0–11.0) was procured from LKB (Stockholm, Sweden). Various ε-N-[methyl-^{14}C]-labeled lysine derivatives were prepared by growing *Neurospora crassa* in the presence of L-[methyl-^{14}C]methionine and by isolating them from the acid hydrolysate of the methyl-^{14}C-labeled protein[6]. N^G-Monomethyl-L-arginine*, N^G,N^G-dimethyl-L-arginine (asymmetric) and N^G,N'^G-dimethyl-L-arginine (symmetric) as their di(4′-hydroxyazobenzene-4-sulfonate) salt monohydrates were obtained from Calbiochem-Behring (La Jolla, CA, U.S.A.). The rest of the compounds were obtained either from Sigma (St. Louis, MO, U.S.A.) or Fisher Scientific (Pittsburgh, PA, U.S.A.) and were of the highest grade available.

* G = Guanidino group.

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Isoelectric focusing (IEF)

IEF of various amino acids and their derivatives was carried out as described by Vesterberg[7] and Farooqui *et al.*[5]. A linear gradient of sorbitol was prepared using Ampholine. Then 15 mg of non-radiolabeled or approximately $1 \cdot 10^5$–$10 \cdot 10^5$ cpm of radiolabeled amino acids were electrofocused in a water-jacketed LKB 8100 column at 20°C and 800 V for 20–22 h. After the run was over, fractions of 1 ml were collected at a flow-rate of 1 ml/min by using a LKB minipump. The pH of each fraction was measured at 20°C using an Orion Research pH meter Model 601A.

The peak positions of radioactive amino acids such as [U-^{14}C]lysine, [U-^{14}C]arginine and [U-^{14}C]histidine and ε-N-[methyl-^{14}C]lysine derivatives were determined by mixing 0.1 ml from each fraction with 5 ml of scintillation fluid Formula 963 (New England Nuclear) and by counting for radioactivity in a Packard Tri-Carb spectrometer. For the remaining compounds, the fractions were appropriately diluted prior to ninhydrin reaction and an aliquot of the dilute solution was treated with ninhydrin[8]. (Ampholine gives a positive color reaction with ninhydrin.) The absorbancy at 575 nm was measured.

RESULTS AND DISCUSSION

IEF of arginine and its N^G-methylated derivatives

Fig. 1 illustrates the separation and determination of the p*I* values of arginine and its guanidino-N-methylated derivatives by the IEF technique. The radioactivity peak represents arginine whereas three N^G-methylarginines are indicated by ninhydrin (A_{575}). It is apparent that substitution of one of the hydrogens in the guanidino

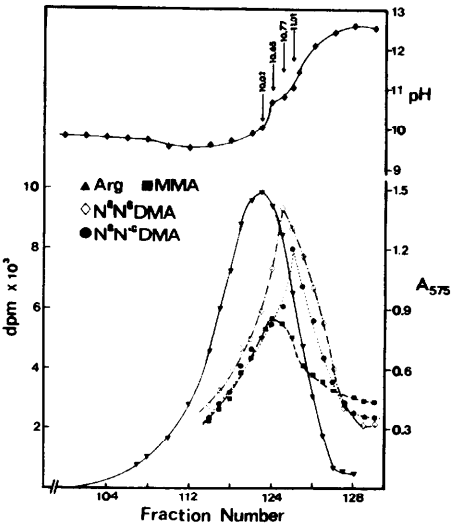

Fig. 1. Isoelectric focusing of arginine and its N^G-methylated derivatives. Approximately $1 \cdot 10^6$ cpm of [U-^{14}C]arginine and 15 mg each of three N^G-methylarginines (MMA = N^G-monomethyl-L-arginine; N^G,N^GDMA = N^G,N^{G}-dimethyl-L-arginine; N^G,N'^GDMA = N^G,N'^G-dimethyl-L-arginine) are electrofocused at 20°C and 800 V for 20–22 h. Because of their closeness, the determination was carried out individually for each compound. Further details of the experimental procedure are in Materials and methods.

group of arginine (N^G-monomethylarginine) increases the basicity by 0.52 pH unit, and an additional substitution further increases it.

pI values of variously methylated amino acids

Table I lists the results of the effect of methyl substitution of a hydrogen atom on the nitrogen (arginine, histidine and lysine), oxygen (tyrosine) or sulfur atoms (cysteine and homocysteine). In all cases except lysine, the methyl substitution increases the p*I* value of the unsubstituted amino acid. In the case of ε-N-methylated lysine derivatives, the changes in p*I* values due to methyl substitution are in good agreement with the values obtainable with ammonia as a model compound[4].

The method commonly employed for the determination of the p*I* value of a compound is based on averaging p*K'* (apparent dissociation constant) values, obtained immediately before and after the formation of an isoelectric species during acid or alkaline titration[9]. However, recently various preparative and analytical isoelectric focusing techniques have been introduced for p*I* determination[10]. Among the preparative IEF methods, Jonsson *et al.*[11] have established a parallelepipedic density gradient column which can be run either horizontally or vertically and the complete IEF run takes only 4 h. This short focusing time is due to a mixed type operation, whereby the column is operated horizontally for a total of 2.5 h, thus ensuring quick

TABLE I

ISOELECTRIC POINTS, p*I*, OF VARIOUSLY METHYLATED AMINO ACIDS

Amino acid and methyl derivatives	*Observed* pI*	*Change from the unsubstituted acid***	*Lit. value*[9]
N-Methyl substitution			
L-Arginine:	10.02 ± 0.08		11.15
N^G-Monomethylarginine	10.54 ± 0.06	+0.52	
N^G,N^G-Dimethylarginine (asymmetric)	10.77 ± 0.03	+0.75	
N^G,N'^G-Dimethylarginine (symmetric)	11.01 ± 0.09	+0.99	
L-Histidine:	7.07 ± 0.05		7.47
3-N-Methylhistidine	7.57 ± 0.01	+0.50	
1-N-Methylhistidine	8.20 ± 0.02	+1.13	
L-Lysine:	9.81 ± 0.02		9.59
ε-N-Monomethyllysine	9.96 ± 0.03	+0.15	
ε-N-Dimethyllysine	9.12 ± 0.01	−0.69	
ε-N-Trimethyllysine	10.24 ± 0.04	+0.43	
O-Methyl substitution			
L-Tyrosine:	5.55 ± 0.03		5.66
O-Methyltyrosine	5.84 ± 0.02	+0.29	
S-Methyl substitution			
L-Cysteine:	10.32 ± 0.01		
S-Methylcysteine	10.40 ± 0.01	+0.08	
L-Homocysteine:	5.65 ± 0.02		5.54
S-Methylhomocysteine (Methionine)	5.74 ± 0.01	+0.09	5.74

* Values reported are mean ± S.D. of four IEF experiments.

** A plus sign indicates an increase and a minus sign a decrease in p*I* value.

steady-state conditions, then slowly rotated to the upright position, where focusing is continued up to a total of 4 h. This column, however, is limited for the analysis of small sample (10–20 mg protein). Another approach called recycling isoelectric focusing, has been described by Egen *et al.*[12]. For analytical purposes, Agarose IEF and Ultrathin IEF have recently been employed[10].

Although these analytical techniques have successfully been used for the analysis of peptides[10] (with a minimum length of fifteen amino acids), to our knowledge no report has appeared which describes the use of IEF for small molecules such as amino acids. The IEF technique used in our study, however, indicated that these relatively small-molecular-weight compounds can easily be electrofocused and their p*I* values can easily be determined. The technique described is simple and satisfactorily reproducible. In most cases, the p*I* values determined by IEF are very close to the literature values which were determined by the titration method. One further advantage of this method is that four or five samples can simultaneously be applied in a single run as long as different criteria for their identication are employed, *e.g.*, by determining ^{3}H and ^{14}C radioactivity, absorbance measurement and ninhydrin color development.

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