Isoelectric points of erabutoxins and monoacyl derivatives of erabutoxin b

Estimation of the pK values of amino groups in erabutoxins by using isoelectric-focusing data

Nobuo UI,* Chikahisa TAKASAKI† and Nobuo TAMIYA†

*Department of Physical Biochemistry, Institute of Endocrinology, Gunma University, Maebashi 371, and

†Department of Chemistry, Faculty of Science, Tohoku University, Sendai 980, Japan

(Received 2 November 1981/Accepted 8 December 1981)

The isoelectric points of erabutoxins a, b and c, neurotoxic proteins of a sea snake, *Laticauda semifasciata*, were determined by density-gradient isoelectric focusing. The same measurement was also made with monoacyl derivatives of erabutoxin b, in which each one of all amino groups had been either acetylated or propionylated. Erabutoxins a and b showed the same isoelectric point at pH 9.68. The values for [1-*N*^α-acetyl-arginine]-, [15-*N*^ε-acetyl-lysine]-, [27-*N*^ε-acetyl-lysine]-, [47-*N*^ε-propionyl-lysine]- and [51-*N*^ε-acetyl-lysine]-erabutoxin b were at pH 9.52, 9.31, 9.45, 9.22 and 9.09 respectively, being definitely different from each other and lower than the value for the unmodified molecule. The isoelectric point of erabutoxin c, which is [51-asparagine]-erabutoxin b, was the same as that of [51-*N*^ε-acetyl-lysine]erabutoxin b. Assuming that no change in pK occurs on monoacylation, the pK values of amino groups in erabutoxin b were calculated from the isoelectric-point data. It is indicated that the pK values of ε-amino groups differ markedly from each other and that the value of α-amino group is anomalously high.

Isoelectric focusing is a method useful for the accurate determination of isoelectric points of proteins, as well as for the effective fractionation of protein mixtures (Svensson, 1962; Vesterberg & Svensson, 1966). As the isoelectric point of a protein depends upon the dissociation constants (pK) of the ionizable groups involved, which, in turn, more or less reflect the tertiary structure of the protein, isoelectric focusing can be of value also as a tool for the conformational studies of proteins. In fact, a definite shift in isoelectric point has been found with several proteins on denaturation caused by urea or on conformational change brought about by the interaction with specific ligands (Ui, 1971*a,b*, 1973). It has been shown that, under favourable conditions, the nature of the dissociable groups responsible for this shift can be predicted, provided that the chemical structure of the protein is known. However, since a large number of dissociable groups contribute to the net charge of a protein in the isoelectric region, unambiguous information on the pK values cannot be obtained generally from the results of isoelectric focusing alone. Although comparison of the isoelectric points of a large variety of derivatives of a protein, in which each of the dissociable groups has been converted into an undissociable group by natural substitution or chemical

modification, is expected to be valuable as an approach to explore pK values, no attempt has yet been made.

Erabutoxins a, b and c are the neurotoxic proteins from the venom of a sea snake, *Laticauda semifasciata*. They are all basic proteins consisting of 62 amino acid residues, and their amino acid sequences have been elucidated (Tamiya & Arai, 1966; Sato & Tamiya, 1971; Endo *et al.*, 1971; Tamiya & Abe, 1972; Maeda & Tamiya, 1977). In erabutoxin a, His-26 of erabutoxin b is replaced by Asn-26, and in erabutoxin c, Lys-51 of erabutoxin b is replaced by Asn-51; thus erabutoxins a and b have four ε-amino groups and one α-amino group within the molecule, whereas erabutoxin c lacks one of the ε-amino groups in other erabutoxins. The three-dimensional structures of erabutoxins in crystals have been also reported (Low *et al.*, 1976; Tsernoglou & Petsko, 1976, 1977; Kimball *et al.*, 1979). The intramolecular states of various amino acid residues in solution have been studied by n.m.r. (Inagaki *et al.*, 1978, 1980). Furthermore, all the five monoacetylated derivatives of erabutoxin b were prepared and their toxicity values compared (Hori & Tamiya, 1976).

The present investigation was undertaken to compare the isoelectric points of erabutoxins a, b

and c and the derivatives of erabutoxin b, in which each one of all amino groups was acetylated or propionylated, in the hope that the pK values of amino groups in erabutoxins might be assessed. Most remarkable was the finding that each of the erabutoxin b derivatives monoacylated at one of the ϵ -amino groups showed a different isoelectric point, suggesting that the pK values of ϵ -amino groups differ markedly from each other. It has been also indicated that the pK value of the α -amino group in erabutoxins is anomalously high.

Materials and methods

Erabutoxins and derivatives

Erabutoxins a, b and c were prepared as described previously (Tamiya & Arai, 1966; Tamiya & Abe, 1972). The amino groups of erabutoxin b were partially acetylated with acetic anhydride, and the reaction products were fractionated by CM-cellulose and Bio-Rex 70 column chromatography according to the procedure reported previously by Hori & Tamiya (1976) to yield monoacetyl derivatives, [1- N^{α} -acetylarginine]-, [15- N^6 -acetyl-lysine]-, [27- N^6 -acetyl-lysine]- and [51- N^6 -acetyl-lysine]-erabutoxin b. Another monoacyl derivative used was [47- N^6 -propionyl-lysine]erabutoxin b, and it was prepared by the reaction of N -succinimidyl propionate with erabutoxin b. The method used for the purification of the derivative and for the location of propionyl groups incorporated was similar to that used for acetylation (Hori & Tamiya, 1976).

Isoelectric focusing

Density-gradient isoelectric focusing was carried out in an LKB column (LKB Produkter AB, Bromma, Sweden) of 110-ml capacity in a manner essentially similar to that described previously (Ui, 1971a). Carrier ampholytes (Ampholine) used for the creation of the pH gradient were purchased from LKB. A linear density gradient in the column was prepared with the aid of an LKB gradient mixer. The dense solution contained 24 g of sorbitol, 1.9 ml of 20% (w/v) Ampholine, pH 9–11, and distilled water to make a final volume of 48 ml, whereas the less dense solution contained 0.3 ml of 20% (w/v) Ampholine, pH 9–11, and 0.2 ml each of 40% (w/v) Ampholine pH 8–10 and pH 6–8 in a final volume of 48 ml. The sample was introduced into the column during the gradient formation, after being dissolved in a small volume of the appropriate mixture of the dense and less dense solutions, to give a narrow protein zone near the middle of the column; hence the protein was never exposed to pH higher than 10. The cathode solution used for the bottom of the column and the central tube consisted of 20 ml of 60% (w/v) sorbitol solution to which 2.0 ml of

1 M-NaOH was added. The anode solution added to the top of the column was 0.01 M-acetic acid.

The column was maintained thermostatically at 5°C. Runs were started at an initial potential of 400 V, and the voltage was increased stepwise to obtain a limiting value of 700 V in 24 h. The total time of electrolysis was 3 days.

On completion of the run, the column was eluted from the bottom at a flow rate of 80 ml/h with the aid of a peristaltic pump. The effluent was monitored with a Hitachi UV-VIS effluent monitor equipped with a flow cell of 2 mm path-length at 280, 260 and 230 nm. Although only the A_{280} was usually used for the determination of protein concentrations, values of other wavelengths were useful to distinguish small protein peaks from peaks due to impurities in carrier ampholytes. Effluent fractions (2 ml) were collected in a fraction collector, and the measurement of pH was made immediately at 22°C; thus the isoelectric points reported in the present paper correspond to those at 22°C (Vesterberg & Svensson, 1966). No correction was made for the effect of solvent compositions on the measured pH, although this may introduce a small error, as reported by Gelsema *et al.* (1978).

Results

Isoelectric focusing of erabutoxins a, b and c

Fig. 1 shows isoelectric-focusing profiles of erabutoxins a, b and c. All the proteins revealed essentially a single peak, although some minor peaks due to impurities were also present.

The isoelectric point determined for erabutoxins a and b was exactly the same and was equal to 9.68. On the other hand, the isoelectric point of erabutoxin c was 9.09, a value definitely lower than that of erabutoxin a or b. These results indicate that His-26 of erabutoxin b is completely deprotonated at the isoelectric pH, being equivalent to Asn-26 of erabutoxin a, and that the presence of Lys-51 in erabutoxins a and b, instead of Asn-51 in erabutoxin c, provides more basic characteristics for the former proteins.

Isoelectric focusing of monoacyl derivatives of erabutoxin b

Derivatives of erabutoxin b in which each one of all the amino groups, i.e., one α -amino and four ϵ -amino groups, was acylated were prepared and subjected to isoelectric focusing. Except for [47- N^6 -propionyl-lysine]erabutoxin b, these were monoacetyl derivatives of erabutoxin b. As shown in Fig. 2, all the monoacyl derivatives showed mainly a single peak.

Table 1 (the column headed 'Observed values') summarizes the isoelectric points determined for monoacyl derivatives of erabutoxin b. The results

Fig. 1. Isoelectric-focusing profiles of erabutoxins a (a), b (b) and c (c)

Carrier ampholytes covering appropriate pH ranges were mixed to create an almost linear pH gradient between 8 and 10 at a final average concentration of approx. 0.5%. About 6 mg of the sample was added to the column and runs were made at 5°C for 3 days. Other conditions are described in the text. Continuous lines indicate the A_{280} recorded continuously with a flow-cell having a light-path of 2 mm. Broken lines show the pH of each fraction collected. The pH of each absorbance peak calculated by interpolation is shown.

for untreated erabutoxins a, b and c are also included. As expected, the isoelectric point of [51- N^6 -acetyl-lysine]erabutoxin b was identical with that of erabutoxin c, which is [51-asparagine]-erabutoxin b. The fact that all the derivatives of erabutoxin b showed different isoelectric points clearly indicates that the pK values for four ϵ -amino- and one α -amino groups in erabutoxin b are not identical with each other.

Estimation of the pK values of amino groups in erabutoxin b

Table 2 lists the numbers of dissociable groups other than amino groups in erabutoxin b, deduced

Fig. 2. Isoelectric-focusing profiles of monoacyl derivatives of erabutoxin b

Monoacyl derivatives of erabutoxin b, i.e., [1- N^{α} -acetylarginine]-, [15- N^6 -acetyl-lysine]-, [27- N^6 -acetyl-lysine]-, [47- N^6 -propionyl-lysine]- and [51- N^6 -acetyl-lysine]-erabutoxin b (α -Ac Eb, 15-Ac Eb, 27-Ac Eb, 47-Prop Eb and 51-Ac Eb) were subjected to isoelectric focusing under the same conditions as in Fig. 1. The abscissa (fraction no.) was adjusted so that each peak can be compared easily with other peaks. The ordinate represents the A_{280} of the effluent in arbitrary units.

from the chemical structure, together with their estimated pK values. One α -carboxy and five side-chain carboxy groups give erabutoxin b and its monoacyl derivatives six total negative charges constantly at their isoelectric pH region (pH 9.09–9.68), since the pK values of carboxy groups are low (Tanford, 1962). Three guanidyl groups provide three positive charges over this pH range due to their high pK values (Tanford, 1962). On the other hand, two imidazole groups will not carry a charge at all over this pH range, because the pK values of His-7 and His-26 in erabutoxin b are reported to be less than 3 and 5.8, respectively, by Inagaki *et al.* (1978) from the results of n.m.r. studies. A single phenolic group (Tyr-25) in erabutoxins is known to be less susceptible to iodination than His-26 (Sato & Tamiya, 1970) and to have a pK value as high as

Table 1. Isoelectric points of erabutoxins a, b and c and monoacyl derivatives of erabutoxin b

Sample	Isoelectric point	
	Observed values	Calculated values*
Erabutoxin a ([26-Asn]erabutoxin b)	9.68	9.68‡
Erabutoxin b†	9.68	
	9.69	
[1- <i>N</i> ^α -Acetylarginine]erabutoxin b	9.52	9.50
[15- <i>N</i> ⁶ -Acetyl-lysine]erabutoxin b	9.31	9.29
[27- <i>N</i> ⁶ -Acetyl-lysine]erabutoxin b	9.45	9.43
[47- <i>N</i> ⁶ -Propionyl-lysine]erabutoxin b	9.22	9.19
[51- <i>N</i> ⁶ -Acetyl-lysine]erabutoxin b	9.09	9.09‡
Erabutoxin c ([51-Asn]erabutoxin b)	9.09	

* Calculated by using the p*K* values of amino groups obtained in the present study (Table 3).

† Observed values from two independent experiments are listed.

‡ Mean values.

Table 2. Dissociable groups in erabutoxin b other than amino groups

The number of each group is expressed as *n_i* and the charge carried by that group at the isoelectric point as *z_i*.

Group	<i>n_i</i>	p <i>K</i>	<i>z_i</i>
α-Carboxy (Asn-62)	1	3.6	-1
Side-chain carboxy	5	4.0-4.5	-5
Imidazole			
(His-7)	1	<3*	0
(His-26)	1	5.8*	0
Phenolic (Tyr-25)	1	12.0*	0
Guanidyl	3	>12	+3
		Σ <i>z_i</i> : -3	

* Taken from Inagaki *et al.* (1978).

12.0 (Inagaki *et al.*, 1978); therefore it will not contribute at all to the charge of isoelectric erabutoxins. From these considerations it is clear that, at their isoelectric points, erabutoxin b and its monoacyl derivatives have three constant negative charges arising from the dissociable groups other than amino groups. This means that, for these proteins to be at isoelectric pH, their amino groups must carry exactly three positive charges in total to compensate for the three negative charges due to other dissociable groups.

The correlation between the pH and the degree of dissociation of ionizable groups on protein is given by the equation of Linderstrøm-Lang:

$$\text{pH} = \text{p}K_{\text{int}}^i + \log [x_i / (1 - x_i)] - 0.868wZ \quad (1)$$

where $\text{p}K_{\text{int}}^i$ refers to the intrinsic dissociation constant of group *i*, *x_i* is the average degree of dissociation of group *i* at any pH, *Z* is the net charge of the protein and *w* is a parameter that accounts for the electrostatic interaction between charged groups (Tanford, 1962). Since, at the isoelectric point (pI),

Z is equal to zero, the above equation is simplified to give:

$$\text{pI} = \text{p}K_{\text{int}}^i + \log [x_i / (1 - x_i)] \quad (2)$$

On the other hand, the average charge, *z_i*, on cationic groups is expressed by $1 - x_i$. Therefore from eqn. (2) the charge on any amino group is expressed by:

$$z_i = 1 / (1 + 10^{\text{pI} - \text{p}K_{\text{int}}^i}) \quad (3)$$

As mentioned above, the sum of the charges on all amino groups at the isoelectric point is equal to three for erabutoxin b and its monoacyl derivatives. Hence:

$$\sum_{\text{amino}} z_i = \sum_{\text{amino}} [1 / (1 + 10^{\text{pI} - \text{p}K_{\text{int}}^i})] = 3 \quad (4)$$

Since we can measure the isoelectric point for six compounds independently, six simultaneous equations in the form of eqn. (4) are obtained. If we assume that the $\text{p}K_{\text{int}}^i$ value for each amino group in erabutoxin b is kept unchanged in all monoacyl derivatives of erabutoxin b, the number of unknown $\text{p}K_{\text{int}}^i$ values in these equations is five, i.e., those for one α-amino and four ε-amino (Lys-15, Lys-27, Lys-47 and Lys-51) groups. The most suitable set of these five variables was obtained by the least-squares method using a computer. The results are summarized in Table 3. Conversely, when five p*K* values thus obtained were utilized to calculate the isoelectric points of erabutoxin b and its monoacyl derivatives on the basis of eqn. (2), agreement between the observed and calculated values was satisfactory, as seen in Table 1.

It is apparent from Table 3 that four ε-amino groups in erabutoxin b have $\text{p}K_{\text{int}}^i$ values different from each other, ranging from 9.44 to more than 11.5. This appears to reflect different microenvironments of different lysine residues in erabutoxin b.

Table 3. Calculated $pK_{int.}$ values for the amino groups in erabutoxin b

Group	Residue	$pK_{int.}$
α -Amino	Arg-1	9.29
ϵ -Amino	Lys-15	9.80
	Lys-27	9.44
	Lys-47	10.29
	Lys-51	>11.5

Interestingly, the $pK_{int.}$ value of the α -amino group, 9.29, is much higher than the value usually accepted as normal (Tanford, 1962), i.e., 7.5.

Effect of urea on the isoelectric point of erabutoxins

On isoelectric focusing of erabutoxins a and b in the presence of 6M-urea, their isoelectric points were determined to be 9.60 and 9.58 respectively after appropriate correction was made for the effect of urea on the measured pH (Ui, 1971a). These values were only slightly lower than the value obtained in the absence of urea, 9.68. A similar small downward shift in isoelectric point was observed when a solution of erabutoxin b was incubated with 8M-urea and then subjected to isoelectric focusing in the absence of urea; incubation for 60min at 37°C was enough for completion of this conversion.

The c.d. spectrum of erabutoxin b (210–250nm) was identical in either the presence or absence of 8M-urea (results not shown). Hence the main-chain conformation of erabutoxin appears to be stable in urea at neutral pH. Although a decrease in isoelectric point of a protein can occur by the reaction of amino groups with cyanate, which may be formed in concentrated urea solutions (Stark *et al.*, 1960), this will not be the case for erabutoxin, because the extent of shift in the isoelectric point of erabutoxin b caused by urea was smaller than that observed with any monoacylated derivative of erabutoxin b. Probably a small and local conformational change, which escaped detection by the c.d. spectrum is responsible for the urea-induced shift in the isoelectric point of erabutoxin. One possible interpretation is that the α -amino group present in the *N*-terminal arginine residue of erabutoxin, having an anomalously high pK value, is normalized in the presence of urea, although other possibilities cannot be ruled out.

Discussion

The amino groups of erabutoxins attract special attention because the sea-snake and cobra neurotoxins are usually basic proteins with rather high lysine and arginine contents. Erabutoxins a and b

have five amino groups in their molecule; these are four ϵ -amino groups located on lysine residues at positions 15, 27, 47 and 51, in addition to one α -amino group at the *N*-terminus. Lys-51 of erabutoxins a and b is replaced by Asn-51 in erabutoxin c. Hori & Tamiya (1976) prepared all the five possible monoacetyl derivatives of erabutoxin b and found that the α -amino group on the *N*-terminus and ϵ -amino groups on lysine residues at positions 15 and 51 could be modified without loss of the toxicity, whereas the acetylation of the ϵ -amino group at position 27 or 47 reduced the toxicity. These results are in accordance with the fact that residues 15 and 51 are variant, whereas residues 27 and 47 are conserved among almost all neurotoxins (Tamiya *et al.*, 1980).

The results of the present isoelectric-focusing experiments have clearly indicated that the isoelectric points of five monoacylated erabutoxin b derivatives, which are naturally less basic than unmodified erabutoxin b, differ definitely from each other (Table 1). Among these monoacylated derivatives, the terminal *N*-acetyl-toxin showed the highest isoelectric point, and the values for Lys-27-, Lys-15-, Lys-47- and Lys-51-acyl derivatives decreased in the order listed. The isoelectric point of [Lys-51-acetyl]erabutoxin b, which was least basic, coincided with that of erabutoxin c, i.e., a natural derivative of erabutoxin b having an asparagine residue instead of Lys-51.

Since all the derivatives used, as well as native erabutoxins, showed the same c.d. spectrum in the u.v. region (Hori & Tamiya, 1976; N. Ui, unpublished work) and all of them, except for the Lys-51-acetyl derivative, were crystallizable (N. Tamiya, unpublished work), there seems to be very little change in the molecular conformation of erabutoxin b on monoacylation of one of the amino groups. The intrinsic pK value for each amino group of erabutoxin b was computed from the isoelectric-focusing data by the procedure mentioned in the Results section (Table 3). The calculation was based on the assumption that each amino group kept its pK value unchanged in all the toxins and derivatives.

Recently Inagaki *et al.* (1980) determined the apparent pK values by their proton n.m.r. studies on erabutoxins, i.e., the observation of the pH-dependence of chemical shifts in an alkaline pH region. Their values are not always in harmony with those of the present investigation. A direct comparison of these values is, however, difficult, owing to the differences in experimental conditions. Their values were obtained in 2H_2O in the presence of salt at 37°C, and the present data in 1H_2O in the absence of salt at 22°C (isoelectric-focusing runs were made at 5°C). Another big difference is that their observations were made by titrating the protein

with alkali until lysine residues were deprotonated ($\text{pH} > 11$), whereas in the present experiments the pH values were kept within the isoelectric region ($\text{pH} 9.09\text{--}9.69$) of the toxins and derivatives. Preliminary experiments showed that treatment of erabutoxin b at pH above 12 induced a small but definite change in its c.d. spectrum, suggesting a conformational change at higher pH . This may partially explain the reason for the discrepancy of the results by both methods and justify the present approach using the data from isoelectric focusing. On the other hand, the observation by n.m.r. that the monoacetylation of Lys-27 in erabutoxin b lowered the pK values of three other ϵ -amino groups (Lys-15, Lys-47 and Lys-51) by about 0.2 apparently contradicts the assumption we made to calculate the pK values from isoelectric-focusing data. However, since the isoelectric point of [Lys-27-acetyl]erabutoxin b was only 0.23 pH unit lower than that of unmodified erabutoxin b, it is difficult to accept the n.m.r. observation as such. Until this problem is settled, the following discussion will have to be taken with some reservation.

The pK values calculated for Lys-27 and Lys-47 residues, which play essential roles in the manifestation of toxicity, probably as the binding sites to the acetylcholine receptor, are quite different from each other, being 9.44 and 10.29 respectively. The former value is much lower than the value usually accepted as normal, i.e., 9.6–10.4 (Tanford, 1962). The reason for this abnormality is not clear, especially since the X-ray-crystallographic data show that Lys-27 is located in the vicinity of the negatively charged group of Glu-38 (Kimball *et al.*, 1979). The n.m.r. measurements also gave the lowest pK value (9.70) to this group among the ϵ -amino groups. Inagaki *et al.* (1980) ascribed this low shift of pK to the involvement of Lys-27 in the core of the tertiary structure of the toxin molecule.

Two other ϵ -amino groups on Lys-15 and Lys-51 residues, which are located on the other side of the erabutoxin b molecule from the 'receptor-binding side', showed pK values of 9.80 and more than 11.5, respectively. The reason for the peculiarly high pK value of Lys-51 remains to be clarified, although it may be related to the low reactivity of the residue on acetylation. It is noteworthy that the higher is the pK value of the ϵ -amino residue (Lys-27, -15, -47 and -51), the lower is the yield of monoacetyl derivative on that amino group (Hori & Tamiya, 1976). There is also an observation with erabutoxin c that the monopropionylated derivatives are eluted from a DEAE-cellulose DE-52 column at $\text{pH} 8.5$ in the order of α -, Lys-27, Lys-15 and Lys-47 derivatives (results not shown). The order coincides with that of pK values (erabutoxin c lacks Lys-51). The results suggest different microenvironment for each ϵ -amino group. On the other hand,

n.m.r. studies gave similar pK values for the ϵ -amino groups of Lys-15, -47 and -51.

The anomalously high pK value of 9.29 for the α -amino group would be accounted for by the presence of Glu-58 in the vicinity. The slight decrease in the isoelectric point of erabutoxin b observed in the presence of urea or after the treatment with urea could occur if the local conformation is changed by the denaturant.

Owing to its extraordinarily high resolving power (Vesterberg & Svensson, 1966), isoelectric focusing has been used widely for the criteria of the homogeneity of purified protein preparations. Several proteins accepted as monodisperse by other conventional methods have been shown to contain several components in isoelectric focusing (Susor *et al.*, 1969). There are various reasons for the microheterogeneity (Williamson *et al.*, 1973). In some cases the presence of multiple components can be ascribed to the variation in the amide and sialic acid contents. However, there are cases in which no such simple explanation is possible. The present finding that the monoacyl derivatives of erabutoxin b with the same numbers of dissociable groups show a variety of isoelectric points is of special interest in this respect. Since the isoelectric point of a protein can be a sensitive measure of the states of dissociable groups involved, it is conceivable that not only the position isomers studied here, but also genetic variants of proteins with neutral amino acid substitution, may be separable from each other by isoelectric focusing under favourable conditions.

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

References

- Endo, Y., Sato, S., Ishii, S. & Tamiya, N. (1971) *Biochem. J.* **122**, 463–467
- Gelsema, W. J., de Ligny, C. L. & van der Veen, N. G. (1978) *J. Chromatogr.* **154**, 161–174
- Hori, H. & Tamiya, N. (1976) *Biochem. J.* **153**, 217–222
- Inagaki, F., Miyazawa, T., Hori, H. & Tamiya, N. (1978) *Eur. J. Biochem.* **89**, 433–442
- Inagaki, F., Tamiya, N. & Miyazawa, T. (1980) *Eur. J. Biochem.* **109**, 129–138
- Kimball, M. R., Sato, A., Richardson, J. S., Rosen, L. S. & Low, B. W. (1979) *Biochem. Biophys. Res. Commun.* **88**, 950–959
- Low, B. W., Preston, H. S., Sato, A., Rosen, L. S., Searl, J. E., Rudko, A. D. & Richardson, J. S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2991–2994
- Maeda, N. & Tamiya, N. (1977) *Biochem. J.* **167**, 289–291
- Sato, S. & Tamiya, N. (1970) *J. Biochem. (Tokyo)* **68**, 867–872

- Sato, S. & Tamiya, N. (1971) *Biochem. J.* **122**, 453–461
- Stark, G. R., Stein, W. H. & Moore, S. (1960) *J. Biol. Chem.* **235**, 3177–3181
- Susor, W. A., Kochman, M. & Rutter, W. J. (1969) *Science* **165**, 1260–1262
- Svensson, H. (1962) *Arch. Biochem. Biophys. Suppl.* **1**, 132–138
- Tamiya, N. & Abe, H. (1972) *Biochem. J.* **130**, 547–555
- Tamiya, N. & Arai, H. (1966) *Biochem. J.* **99**, 624–630
- Tamiya, N., Takasaki, C., Sato, A., Menez, A., Inagaki, F. & Miyazawa, T. (1980) *Biochem. Soc. Trans.* **8**, 753–755
- Tanford, C. (1962) *Adv. Protein Chem.* **17**, 69–165
- Tsernoglou, D. & Petsko, G. A. (1976) *FEBS Lett.* **68**, 1–4
- Tsernoglou, D. & Petsko, G. A. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 971–974
- Ui, N. (1971a) *Biochim. Biophys. Acta* **229**, 567–581
- Ui, N. (1971b) *Biochim. Biophys. Acta* **229**, 582–589
- Ui, N. (1973) *Ann. N.Y. Acad. Sci.* **209**, 198–209
- Vesterberg, O. & Svensson, H. (1966) *Acta Chem. Scand.* **20**, 820–834
- Williamson, A. R., Salaman, M. R. & Kreth, H. W. (1973) *Ann. N.Y. Acad. Sci.* **209**, 210–224