Acta Chemica Scandinavica B 37 (1983) 817–822

The Nucleophilic Reactivity of Valine Methylamide. A Model of the *N*-Terminal Valine Residues of Hemoglobin

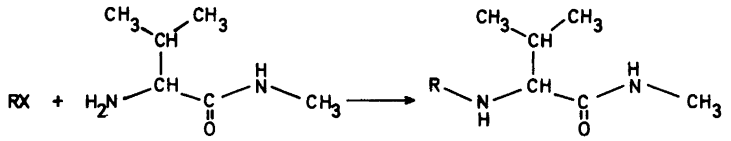
VERONIQUE POIRIER [a] and CARL JOHAN CALLEMAN [b]

[a] Pavillon Pasteur, Institut Curie, 26, Rue d'Ulm, 75231 Paris, France and [b] Department of Radiobiology, University of Stockholm, S-106 91 Stockholm, Sweden

Valine methylamide (*1*) was synthesized as a model of the *N*-terminal valine residues of hemoglobin and a separation system was devised for its reaction products with a few mutagenic/carcinogenic alkylating agents. The pK'_a of *1* was 7.65 at 37 °C or about one pH unit higher than those previously determined for the *N*-terminal valine residues of liganded hemoglobin, in line with the existence of salt bridges between these residues and negatively charged amino acids. The nucleophilicity, *n*, of *1* in the Swain-Scott scale was about 4.35, which in combination with a relatively low pK_a-value in comparison to other aliphatic amines makes it the most reactive model of nucleophilic amino groups in proteins at physiological pH. The reactivity *versus* the *N*-terminal valine residues of hemoglobin of the few, relatively small, alkylating agents studied to date were slightly lower than predicted from their reactivity *versus* *1* also when the Brønsted dependence of the nucleophilicity on the basicity was taken into account.

The primary amino groups of the *N*-terminal valine residues of the α- and β-chains are two reactive nucleophilic sites of the hemoglobin molecule. Reactions at these sites with monosaccharides lead to the formation of glycosylated hemoglobins,[1] which are believed to be formed by thermal (enzymatically uncatalyzed) reactions.[2] Moreover, reactions with carbon dioxide at these sites play an important role in the allosteric interactions of hemoglobin[3] and their carbamoylation by potassium cyanate has been suggested as a cure for sickle cell anemia. Also electrophilic compounds with mutagenic/carcinogenic effects react with the *N*-terminal valine residues of hemoglobin, and a method for dose monitoring of such compounds *in vivo* by determining their reaction products with the *N*-terminal valine residues of hemoglobin, has been suggested.[4] Due to this link to dose monitoring *in vivo* we are projecting the use of valine methylamide (*1* in Fig. 1) as a dose monitor (trapping agent) for electrophilic reagents, RX, in mutagenicity test systems *in vitro*.

In order to investigate the suitability of *1* as a dose monitor in *in vitro* test systems we have determined its nucleophilic reactivity *versus* a few mutagenic/carcinogenic electrophiles and compared it with the corresponding reactivity of the *N*-terminal valine residues of hemoglobin.



1

Fig. 1.

0302-4369/83 $2.50

MATERIALS AND METHODS

Chemicals. *1* was prepared by dissolving 9 g of L-valine methyl ester (Sigma, S:t Louis, Mo.) in 25 ml of 40 % methylamine (Merck, *p.a.*) and leaving it to react for 15 h at room temperature. The reaction mixture was evaporated to dryness in a rotatory evaporator at 60 °C and the syrup dissolved in 5 ml of water, which was applied to a 3.5×10 cm Dowex 1 column in the OH^--form. The uncharged *1* was collected in 200 ml of water effluent, whereas the negatively charged valine, produced by hydrolysis of the valine methyl ester, was retained on the positively charged column. Upon evaporation, the water effluent yielded a residue which resisted attempts of recrystallization in different organic solvents, but was free from ninhydrin-positive contaminants when analyzed on TLC (1-propanol—H_2O, 7:3, SiO_2). Mass spectra were obtained using the direct inlet probe of a Finnigan 4021 mass spectrometer. The CI spectrum with methane as the reagent gas revealed ions with m/z=131, 159, 171 as expected for a compound of molecular weight 130. The electron impact spectrum displayed fragments corresponding to M, M−CH_3, M−$CONHCH_3$, and M−C_3H_7, and gave a larger peak of M+1 than of M, which at moderate sample pressures is characteristic of amines and amides.[5] [^{14}C]Methyl methanesulfonate (2.2 GBq/mmol), [U-^{3}H]ethyl methanesulfonate (925 MBq/mmol), [U-^{14}C]ethylene oxide (800 MBq/mmol) and [7-^{3}H]styrene oxide (3.3 GBq/mmol) were purchased from the Radiochemical Centre, Amersham, England. *N*-Acetoxy-*N*-acetyl-9-aminofluorene-[ring-^{3}H] (AAAF) (36.9 GBq/mmol) was a kind gift from Dr. R. Pero.

Determination of rate constants. The reactions between the alkylating agents and *1* were performed in 1 ml of water solution in sealed pyrex tubes equipped with screw caps in a thermostated water bath at 37 °C. The 0.05 M solutions of *1* were adjusted to pH=7.4 by addition of hydrochloric acid. About 37 kBq of the electrophiles were used providing for at least a thousandfold excess of the concentration of *1* in relation to the electrophiles. The reaction time was 60 min for all electrophiles studied. The reactions were terminated by addition of 1 ml of 1 M hydrochloric acid.

The reaction mixtures with methyl methanesulfonate, ethyl methanesulfonate, ethylene oxide and styrene oxide were applied to Dowex 50 ion exchange columns (12×1 cm, Na^+-form), which were washed with 100 ml of water to remove excess radiolabeled reagent followed by elution with a 0.03 M sodium phosphate buffer at pH=8.25 with a flow rate of about 85 ml per hour. Unreacted *1* eluted after 180 ml of buffer and was detected by ninhydrin on TLC. The relative retention volumes of the different alkylated derivatives of *1* are shown in Table 1.

The reaction mixtures with AAAF were separated on a reverse phase column (Spherisorb ODS, 5μ, Jones Chromatographs Ltd.) on a high performance liquid chromatograph (Laboratory Data Control) with a 45 min gradient from 20 to 100 % methanol in water. The reaction product of *1* with AAAF eluted after 22 min.

The fractions taken out from the separations were counted for radioactivity in an Intertechnique SL 30 scintillation spectrometer after mixing 1 ml from each fraction with 2 ml of Lumagel.

Reaction Kinetics. The concentrations of the reaction products, |RY|, between *1* and the different alkylating agents were determined by radioactivity counting. The second order rate constants, k, were calculated according to the formula

$$k = \frac{|RY|\ k'}{|Y|_0|RX|_0 t\ (1-e^{-k't})}$$

where the starting concentrations of the alkylating agent and *1* are denominated $|RX|_0$, and $|Y|_0$, respectively, and the term $k'/(1-e^{-k't})$ corrects for the decrease of the concentration of the alkylating agent during the reaction. The first order rate constant for the disappearance of the alkylating agent, k', was estimated from the sum of its uncorrected rate of reaction with *1*, and with water.[6] For methyl methanesulfonate k' was estimated to 75×10^{-6} s^{-1}, whereas it was considerably lower for the other alkylating agents.

The values for the nucleophilicity, n, of *1* were calculated according to the equation of Swain and Scott[7] $\log (k_Y/k_{H_2O}) = sn$ where k_Y and k_{H_2O} are the second order rate constants for the reactions between the alkylating agents and Y and water, respectively, and s, is the substrate constant which expresses the sensitivity of the alkylating agent to the nucleophilicity, n, of the nucleophile.

Determination of the pK_a'-value of 1. The pK_a' of *1* was determined at 37 °C with a Titrator TTT2

Table 1. Retention volumes relative to that of valine methylamide on a 12×1 cm Dowex 50 column eluted with 0.03 M phosphate buffer at pH=8.25.

Valine methylamide	1.00 (=def.)
N^2-Methylvaline methylamide	1.17±0.03
N^2-Ethylvaline methylamide	1.20±0.03
N^2-(2-Hydroxyethyl)valine methylamide	0.29±0.01
N^2-(2-Hydroxy-2-phenylethyl)-valine methylamide	1.17±0.01

pH-meter (Radiometer, Copenhagen) equipped with an autoburette. A solution of 0.025 mmol of *1* was prepared in 25 ml of 1 mM HCl and the pH was measured after additions of small volumes of a solution of 0.1 M NaOH (P.H. Tamm, Uppsala, Sweden). The pK'_a-value 7.65 of *1* was obtained graphically by plotting ΔV NaOH/ΔpH as a function of pH.

RESULTS AND DISCUSSION

The retention volumes of a few alkylated derivatives of *1* relative to that of the compound itself are shown in Table 1. Since the α-amino group is the only ionizable group in the alkylated derivatives of *1*, the only factors which are expected to influence the chromatographic behavior of these derivatives are the p*K*-values of their amino groups and their lipophilicity. This makes it possible to obtain good separations between compounds with small differences in p*K*-values by eluting them with buffers which have a pH slightly above these p*K*-values and a low ionic strength.

The electron-affinity of the alkyl group introduced onto *1* seems to explain the chromatographic behavior observed. Electron-donating groups like methyl and ethyl groups increase the p*K*-values of the amino groups and consequently the retention volumes on cation exchangers of the corresponding derivatives of *1*, whereas electron-attracting groups like the 2-hydroxyethyl group have an inverse effect on the retention. The (2-hydroxy-2-phenylethyl) group introduced onto *1* by the reaction with styrene oxide is an electron-attracting group, but the corresponding derivative of *1* is, similarily to the common aromatic amino acids, retarded on Dowex ion-exchangers due to its lipophilicity.

The reaction product between *1* and the carcinogen AAAF was chromatographed on a reverse phase column since it was irreversibly retained on the Dowex ion exchanger due to its aromatic structure. Despite the fact that its rate constant with the *N*-terminal residues of hemoglobin has yet to be determined, it was included in the study to demonstrate the feasibility of monitoring also aromatic reaction products with *1*.

The second order rate constants for the reactions of a few electrophilic reagents with *1* at pH=7.4 and 37 °C are listed in Table 2. Also included in Table 2 are the mean values of the rate constants for the *N*-terminal valines of the α- and β-chains of hemoglobin. The rate constants of *1* were determined as described above, whereas those of the hemoglobins, taken from other sources, were determined in red blood cells treated *in vitro* at 37 °C with the electrophilic reagents. Since the pH within the red blood cells has been determined to 7.40[8] the rate constants should be strictly comparable.

Table 2. Second order reaction rate constants, $10^3 \times (k \pm \text{S.D.})$ ($M^{-1}s^{-1}$) at 37 °C and *pH*=7.4 for the reactions of a few electrophilic reagents with valine methylamide and the mean of the rate constants of the *N*-terminal valine residues of the α- and β-chain of hemoglobin, respectively.

Electrophile	Valine methylamide	Hemoglobin		
		Mouse	Rat	Human
Methyl methanesulfonate	1.28±0.16 (n=3)[a]		0.9[b]	0.5–1.0[b]
Ethyl methanesulfonate	0.040±0.004 (n=3)[a]	0.030[c]		
Ethylene oxide	0.20±0.0006 (n=4)[a]	0.1[d]		0.2[e]
Styrene oxide	0.28 (n=2)[a]			
N-Acetoxy-*N*-acetyl-aminofluorene	0.065±0.019 (n=10)[a]			

[a] n=number of determinations. [b] Ref. 9. [c] Ref. 10. [d] Ref. 11. [e] Ref. 12.

The pK-values at 25 °C of the N-terminal valine residues of the α- and β-chains of human hemoglobin have been estimated to about 6.8 and 7.0 in the liganded state and 7.8 and 6.8, respectively, in the deoxy state,[13] to be compared with 7.65 of the model nucleophile *1* at 37 °C or 8.0 of valine amide at 25 °C.[14] It can thus be concluded that the tertiary structure of hemoglobin decreases the pK of its N-terminal groups by about 1 unit except for in the case of valine 1α in the deoxy state. This observation lends additional support to the suggestion that the N-terminal valine residues are electrostatically bound to negatively charged groups in the tetrameric hemoglobin complex and may play important roles in the allosteric properties of this protein.[15]

If the rate constants in Table 2 are divided by the degrees of dissociation of the nucleophiles at pH=7.4 (0.36 for *1* and 0.85 for the oxyhemoglobins assuming a decrease in the pK-value of 0.3 units when the temperature is increased to 37 °C from 25 °C) the values for the pH-independent, intrinsic, rate constants, k_{int}, in Table 3 are obtained. If the intrinsic rate constants of *1*, k_{int}, are inserted together with k_{H_2O}, the second order rate constants of water and s, the substrate constant, into the equation $\log k_{int}/k_{H_2O}=sn$ of Swain and Scott (7) then it is possible to calculate the nucleophilicity, n, of *1*. The values for k_{H_2O} and s of the compounds studied have been taken from Refs. 6 and 16. The nucleophilicity of *1* calculated from its rate of reaction with methyl methanesulfonate, ethylene oxide and styrene oxide gave similar results (4.48, 4.26 and 4.35), whereas when estimated from its rate of reaction with ethyl methanesulfonate it was about 0.6 units lower, 3.7 (Table 3). This lower nucleophilicity of *1* displayed in the reaction with ethyl methanesulfonate parallels that of other amines in their reactions with the same agent.[17,18]

The determination of the nucleophilicity, n, and pK-value of *1* permits us to compare its nucleophilic reactivity at pH=7.4 with that of a few other models for nucleophilic groups in proteins. The nucleophilicity and pK-value of N^2-acetyl-histidine methylamide, a model for histidine residues in proteins, have been determined to n=3.35[18] and pK=6.31[20] at 37 °C. Despite the higher degree of dissociation of this nucleophile in comparison to *1*, its lower nucleophilic strength makes it roughly four times less reactive than *1* at pH=7.4 in reactions with alkylating agents with substrate constants close to one, such as methyl methanesulfonate and ethylene oxide.[18] In this comparison the nucleophilic reactivity of N^2-acetyl-histidine methylamide was based on the sum of the reactivities of its two imidazole nitrogens. Data are lacking for the nucleophilic reactivity of N^2-acetyl-lysine methylamide, but butylamine with an n-value of 5.13 and a pK-value of 10.63[21] also provides a good model for the ε-amino groups of lysine residues in proteins. Due to its high pK-value, the nucleophilic reactivity of this compound at pH=7.4 is about six hundred times less than that of *1* in reactions with alkylating agents with substrate constants close to one, despite the fact that it is a stronger nucleophile than *1* in the base form. Likewise, the high pK-value of the guanidino group of arginine residues (>12, Ref. 22) gives this group a poor reactivity at pH=7.4. This comparatively high reactivity of *1* at pH=7.4 makes it a promising dose monitor for mutagenic electrophilic reagents in *in vitro* test systems.

It can be concluded that the amino groups of N-terminal valine (or other amino acid) residues

Table 3. Intrinsic rate constants, $k_{int} \times 10^3$ ($M^{-1}s^{-1}$) at 37 °C for the reactions between valine methylamide and a few alkylating agents together with their substrate constants, s, rate constants of hydrolysis, $k_{hydrolysis} \times 10^3$ (s^{-1}) and the nucleophilicities, n, estimated from the reactions with the respective nucleophiles.

Electrophile	k_{int}	$k_{hydrolysis}$	s	n
Methyl methanesulfonate	3.56±0.44	0.020[a]	0.89[b]	4.48
Ethyl methanesulfonate	0.112±0.011	0.017[a]	0.69[b]	3.70
Ethylene oxide	0.56±0.018	0.0025[a]	0.96[b]	4.26
Styrene oxide	0.78	0.013[a]	0.81[c]	4.35

[a] Ref. 16. [b] Ref. 6. [c] Ref. 19.

Table 4. pH-independent rate constants, (k_{int}±S.D.) × 10^3 ($M^{-1}s^{-1}$) at 37 °C for valine methylamide and the *N*-terminal valine residues of oxyhemoglobin with a few electrophilic reagents.

Electrophile	Valine methylamide	Hb		
		Mouse	Rat	Human
Methyl methanesulfonate	3.56±0.44		1.0	0.6–1.2
Ethyl methanesulfonate	0.112±0.011	0.035		
Ethylene oxide	0.56±0.018	0.1		0.25

are the most reactive nucleophilic amino groups in proteins at physiological pH, except in situations where the tertiary structure of a protein lends exceptional reactivities to specific nucleophilic amino acid residues. Differences in the reactivity of alkylating agents with, on the one hand, *N*-terminal valine residues such as in hemoglobin and, on the other hand *1*, should be ascribed to the microenvironment of the *N*-terminal valine residue and/or to the distribution of the alkylating agent within the protein.

As can be seen in Table 4, the mean pH-independent rate constants of the *N*-terminal valine residues of hemoglobin are 4–5 times lower than that of the model compound *1*. Assuming a slope, β, in the Brønsted equation $\log (k_{Y'}/k_{Y''})=\beta(pK_{Y'}-pK_{Y''})$ of 0.2 for the alkyl methanesulfonates [6] and 0.32 for the epoxides [23] a difference in rate constants of a factor of two would be expected for the alkyl methanesulfonates and ethylene oxide, respectively.

It thus seems that the reactivity *versus* electrophilic reagents of the *N*-terminal valine residues of oxyhemoglobin is slightly lower than predicted from their reactivity *versus 1* even if the Brønsted dependence of the nucleophilicity upon the basicity of amino groups is taken into account. Due to the paucity of reaction kinetic data for the *N*-terminal valine residues of hemoglobin this comparison is limited to relatively small alkylating agents and the difference in reactivity may be even more pronounced for bulky reagents. In fact, albeit the *N*-terminal valine residues of human hemoglobin have been shown to be reactive *versus* small electrophilic reagents such as carbon dioxide,[3] potassium cyanate,[13] methyl methanesulfonate[9] and ethylene oxide,[4] the absence of α-chains glycosylated in that position seems to indicate a steric hindrance against reactions on the *N*-terminal valine of the α-chain. This sterical hindrance is presumably exerted by a salt bridge to the *C*-terminal carboxylate group of arginine 141 of the opposite α-chain.[15]

Acknowledgements. We are grateful to Prof. Lars Ehrenberg and Dr. Siv Osterman-Golkar for valuable discussions. The financial support of the Swedish Cancer Society, the Nordic Society against Painful Experiments on Animals, the Hierta-Retzius Scholarship Fund and *Éléctricité de France* is gratefully acknowledged.

REFERENCES

1. McDonald, M. J., Shapiro, R., Bleichman, M., Solway, J. and Bunn, H. F. *J. Biol. Chem. 253* (1978) 2327.
2. Gabbay, K. H., Hasty, K., Breslow, J. L., Ellison, R. C., Bunn, H. F. and Gallop, P. M. *J. Clin. Invest. 44* (1977) 859.
3. Kilmartin, J. V. and Rossi-Bernardi, L. *Physiol. Rev. 53* (1973) 836.
4. Jensen, S., Törnqvist, M. and Ehrenberg, L. In *Proceedings of the American-Swedish Workshop on Individual Susceptibility to Genotoxic Agents in the Human Population,* Raleigh, N. C. 1982.
5. McLafferty, F. W. *Interpretation of Mass Spectra,* University Science Books, Mill Valley, CA 1980.
6. Osterman-Golkar, S. *Thesis,* University of Stockholm, Stockholm 1975.
7. Swain, C. G. and Scott, C. B. *J. Am. Chem. Soc. 75* (1953) 141.
8. Brown, F. F., Campbell, I. D., Kuchel, P. W. and Rabenstein, D. C., *FEBS. Lett. 82* (1977) 12.
9. Nguyen, L. *et al. To be published.*
10. Murthy, M. S. S. *et al. To be published.*
11. Segerbäck, D. *To be published.*
12. Segerbäck, D. *Unpublished.*
13. Garner, M. H., Bogardt, R. A., Jr. and Gurd, F. R. N. *J. Biol. Chem. 250* (1975) 4398.

14. Ghazarian, J. G. *Arch. Biochem. Biophys. 150* (1972) 72.
15. Perutz, M. F. *Nature 228* (1970) 726.
16. Ehrenberg, L. and Hussain, S. *Mutat. Res. 86* (1981) 1.
17. Walles, L. *Thesis,* Stockholm University, Stockholm 1975.
18. Calleman, C. J. and Poirier, V. *Acta Chem. Scand. B 37* (1983) 809.
19. Osterman-Golkar, S. *To be published.*
20. Tanokura, M., Tasami, M. and Miyazawa, T. *Chem. Lett.* (1978) 739.
21. Koskikallio, J. *Acta Chem. Scand. 23* (1969) 1490.
22. Tanford, C. and Hauenstein, J. D. *J. Am. Chem. Soc. 78* (1956) 5287.
23. Hudson, R. F. and Loveday, G. *J. Chem. Soc.* (1962) 1068.

Received December 23, 1982.