Biochemistry

 Volume 23, Number 4 February 14, 1984

Articles

Active Site of C3a Anaphylatoxin: Contributions of the Lipophilic and Orienting Residues†

Cecilia G. Unson, Bruce W. Erickson,* and Tony E. Hugli

ABSTRACT: Activation of the serum complement cascade generates C3a anaphylatoxin, a primary mediator of inflammation. The active-site pentapeptide from the COOH terminus of C3a, Leu-Gly-Leu-Ala-Arg (residues 73–77), exhibits the inflammatory activities and specificity of the native 77-residue polypeptide. Functionally important features of this active site were studied by testing the ability of 22 synthetic analogues of this pentapeptide to contract isolated muscle strips from guinea pig ileum and to desensitize this tissue to contraction induced by human C3a or C5a. The C3a receptors on mast cells and basophils probably contain lipophilic groups that interact with the lipophilic side chains of Leu-73 and Leu-75 and charged groups that interact with the carboxylate and guanidinium groups of Arg-77. The lipophilic contribution of Leu-73 is modest and sterically nonspecific while that of Leu-75 is substantial and sterically specific. Gly-74 and Ala-76 appear to position and orient the adjacent residues Leu-73, Leu-75, and Arg-77 for optimal receptor binding. The contribution of Gly-74 is neither conformationally nor sterically specific while that of Ala-76 is both conformationally and sterically specific. The cellular C3a receptors evidently interact most efficiently with peptides ending in -Leu-Ala-Arg-OH.

Activation of the complement system of human serum results in cleavage of C3, the third complement component, into the activation peptide C3a (M_r 8900) and the activated protein C3b (M_r 171 000) (Hugli & Müller-Eberhard, 1978). Covalent attachment of C3b to receptive surfaces (Law et al., 1979) such as immune complexes or cell surfaces occurs through its metastable binding site, which might contain a reactive 15-membered thiolactone ring (Tack et al., 1980; Khan & Erickson, 1981, 1982). Bound C3b stimulates the phagocytosis of cells and immune complexes and plays an essential role in activation of the complement proteins C3 and C5.

The activation peptide C3a is also an important humoral factor in host defense. Human C3a is a potent mediator of acute inflammatory reactions. At a concentration of 10^{-9} M it induces histamine-mediated contraction of guinea pig ileal tissue in vitro (Hugli & Müller-Eberhard, 1978), and at a dose of 10 pmol it increases the vascular permeability of human skin in vivo (Lepow et al., 1970). C3a is described as an anaphylatoxin because it is chemically and biologically similar to C5a anaphylatoxin, which induces a systemic response in guinea pigs similar to acute anaphylactic shock. Human C3a contains 77 residues in a single polypeptide chain (Hugli, 1975). The six cysteinyl residues form three disulfide bonds that tie the central half of the chain into a knot while both terminal regions form α helices (Huber et al., 1980). C3a is rapidly inactivated in plasma by the action of carboxypeptidase N (EC 3.4.12.7), which removes the COOH-terminal arginyl residue (Bokisch & Müller-Eberhard, 1970). Since the resulting 76-residue peptide exhibits no inflammatory activity, Arg-77 is essential for activity.

We have studied the active site of C3a by synthesizing and assaying a series of peptide segments and analogues based on the COOH terminus of human C3a. The COOH-terminal octapeptide exhibits all of the inflammatory activities and the specificity of native C3a (Hugli & Erickson, 1977). The COOH-terminal pentapeptide, Leu-Gly-Leu-Ala-Arg, is the shortest C3a segment with significant activity (Caporale et al., 1980). This segment, which is present at the COOH terminus of C3a from man (Hugli, 1975), pig (Corbin & Hugli, 1976), rat (Jacobs et al., 1978), and mouse (Domdey et al., 1982), is useful as a model of the C3a active site.

† From The Rockefeller University, New York, New York 10021 (C.G.U. and B.W.E.), and Department of Molecular Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037 (T.E.H.). *Received August 9, 1983.* Preliminary reports of part of this work were presented at the Sixth and Seventh American Peptide Symposia (Unson et al., 1979; Erickson et al., 1981). This work was supported by Public Health Service Grants HL 16411, HL 19795, and HL 25658 from the National Heart, Lung, and Blood Institute, AI 18362 from the National Institute of Allergy and Infectious Diseases, and RR 07065 from the Division of Research Resources, DHHS.

0006-2960/84/0423-0585$01.50/0

In a companion study[1] we have established that the guanidinium and carboxylate groups of Arg-77 are essential for activity (Erickson et al., 1980, 1981). The present study describes several contributions of the preceding four active-site residues (Leu-73, Gly-74, Leu-75, and Ala-76) to spasmogenic activity. Synthesis and assay of 22 single-substitution analogues of the active-site pentapeptide suggest that interaction of the large, lipophilic residues Leu-73 and Leu-75 with cellular C3a receptors and proper positioning and orientation of these residues relative to Arg-77 by the small, hydrophilic residues Gly-74 and Ala-76 are important for activity.

Experimental Procedures

Peptide Synthesis. Peptides were chemically synthesized by the solid-phase method (Erickson & Merrifield, 1976) as described (Caporale et al., 1980), except that a manual peptide shaker was used for agitation and the peptides were purified by reverse-phase liquid chromatography on octadecyl-silica (μBondapak C_{18}, Waters Associates). Each peptide was examined for homogeneity by thin-layer chromatography on silica gel, by analytical reverse-phase liquid chromatography on octadecyl-silica using methanol/acetic acid/water (250:1:750) for isocratic elution, and by amino acid analysis after acid hydrolysis.

Bioassays for Anaphylatoxin Activity. Each peptide was assayed for the ability to contract isolated strips of smooth muscle from guinea pig ileum by a modification (Caporale et al., 1980) of the method of Cochrane & Müller-Eberhard (1968). Tissue desensitization (tachyphylaxis) was measured by examining the ability of purified samples of human C3a (Hugli, 1975) or C5a (Fernandez & Hugli, 1976) to induce contraction of an ileal strip previously exposed to the synthetic peptide and vice versa.

Results

Synthetic Peptides. Twenty-two single-substitution analogues of Leu-Gly-Leu-Ala-Arg, COOH-terminal pentapeptide **1** from C3a anaphylatoxin, were synthesized by the solid-phase method and purified by reverse-phase liquid chromatography. Each peptide was homogeneous by thin-layer chromatography and reverse-phase liquid chromatography and provided acceptable amino acid ratios after acid hydrolysis (data not shown). Amino acid substitutions were made systematically at positions 73–76 in order to elucidate the functionally important features of their side chains. Analogues **5**, **7**, **18**, **20**, and **21** have been described previously (Caporale et al., 1980; Hugli et al., 1983).

Contraction of Smooth Muscle. Each active-site analogue was assayed for the ability to contract isolated strips of smooth muscle from guinea pig ileum. Table I records the chemical structure of each analogue, the minimum effective dose needed to produce full contraction, the relative molar activity compared to that of peptide **1**, and the ability of the analogue to induce desensitization (tachyphylaxis) of the tissue toward contraction induced by human C3a. None of these 23 peptides desensitized the muscle strip toward contraction induced by human C5a. Peptide **1**, which produces full contraction at doses as low as 21–28 nmol, is 500 times less active on a molar basis than native human C3a (Caporale et al., 1980).

Leucine-73. Replacement of Leu-73 in peptide **1** by tyrosine (**2**) or phenylalanine (**3**) actually increased the contractile activity by about 50%. Replacement by isoleucine (**4**) or valine (**5**) produced no significant loss in activity. Replacement by methionine (**6**) or alanine (**7**) decreased the activity by 2-fold or 5-fold, respectively. Thus, Leu-73 can be substituted by an aromatic or large aliphatic amino acid with little or no loss in ileal contractile activity.

Table I: Smooth Muscle Contractile Activity of 23 Synthetic Pentapeptide Analogues from the COOH Terminus of C3a

peptide code	amino acid sequence 73 74 75 76 77	minimum effective dose[a]	relative molar activity	desensitization to C3a
1	Leu-Gly-Leu-Ala-Arg	21–28	[100]	+
2	Tyr-Gly-Leu-Ala-Arg	12–18	160	+
3	Phe-Gly-Leu-Ala-Arg	15–20	140	+
4	Ile-Gly-Leu-Ala-Arg	21–28	100	+
5	Val-Gly-Leu-Ala-Arg	23–28	90	+
6	Met-Gly-Leu-Ala-Arg	40–53	50	+
7	Ala-Gly-Leu-Ala-Arg	110–150	20	+
8	Leu-Ala-Leu-Ala-Arg	6–12	270	+
9	Leu-Ser-Leu-Ala-Arg	40–50	55	+
10	Leu-Val-Leu-Ala-Arg	130–330	10	+
11	Leu-Leu-Leu-Ala-Arg	210–250	10	+
12	Leu-Gln-Leu-Ala-Arg	>450	<5.5	±
13	Leu-Glu-Leu-Ala-Arg	>1020	<2.4	+
14	Leu-Gly-Ile-Ala-Arg	220–250	10	+
15	Leu-Gly-Met-Ala-Arg	1150–1190	2.1	+
16	Leu-Gly-Phe-Ala-Arg	>1640	<1.5	−
17	Leu-Gly-Val-Ala-Arg	>2240	<1.1	±
18	Leu-Gly-Ala-Ala-Arg	>1750	<1.4	−
19	Leu-Gly-Leu-Pro-Arg	600–800	3.5	+
20	Leu-Gly-Leu-Gln-Arg	840–910	2.8	+
21	Leu-Gly-Leu-Ser-Arg	2700–4800	0.7	+
22	Leu-Gly-Leu-Gly-Arg	>420	<5.8	−
23	Leu-Gly-Leu-Glu-Arg	>940	<2.6	−

[a] Minimum concentration of peptide needed to elicit full contraction of a guinea pig ileal strip in a 1.5-mL bath. The notation (>) means that no contraction was seen at that dose.

Glycine-74. Substitution of Gly-74 in peptide **1** by alanine gave analogue **8**, which was nearly 3 times as active as peptide **1**. The Ser-74 analogue (**9**) was half as active as peptide **1** and the Val-74 analogue (**10**) and Leu-74 analogue (**11**) were 10% as active. The Gln-74 analogue (**12**) and Glu-74 analogue (**13**) showed no contractile activity at 20 and 40 times the minimum dose needed for peptide **1**, respectively. These results suggest that position 74 requires a small residue for optimal activity.

Leucine-75. Replacement of Leu-75 in peptide **1** by isoleucine (**14**) caused a 10-fold decrease in contractile activity. More striking, replacement by methionine (**15**) produced a 50-fold decrease. Both of these analogues desensitized muscle strips to contraction by C3a. The larger Phe-75 analogue (**16**) and the smaller Val-75 analogue (**17**) and Ala-75 analogue (**18**) showed no contractile activity at 65–90 times the minimum dose needed for peptide **1**. At these doses, analogues **16** and **18** failed to desensitize muscle strips to later stimulation by intact C3a. Thus, position 75 shows a relatively strict requirement for leucine.

Alanine-76. Substitution of Ala-76 in peptide **1** by proline (**19**) or glutamine (**20**) gave analogues that were about 3% as active as peptide **1**. The Ser-76 analogue (**21**) was only about 0.7% as active. Each of these peptides exhibited desensitization to C3a. The Gly-76 analogue (**22**) and Glu-76 analogue (**23**) were inactive at 17 and 38 times the minimum dose for peptide **1**, respectively, and failed to induce desensitization to C3a. Thus, position 76 shows a strong requirement for alanine.

Discussion

C3a anaphylatoxin is a potent mediator of inflammation

[1] K. F. Fok, L. H. Caporale, J. Volk-Weiss, B. W. Erickson, and T. E. Hugli, unpublished results.

known to produce degranulation of mast cells and basophils, contraction of smooth muscle, and increase in vascular permeability (Hugli & Müller-Eberhard, 1978). C3a also exhibits potent immunosuppressive activities in vitro and may play a significant role in immune regulation in vivo (Morgan et al., 1982; Weigle et al., 1982). Since this humoral factor expresses so many bioactivities of potential importance for physiology and pathophysiology, we have carried out a detailed analysis of the functionally important features of its active site and indirectly of its cellular receptor. The shortest COOH-terminal segment of human C3a to show significant contractile and permeability activities was initially characterized as eight or fewer residues (Hugli & Erickson, 1977) and later defined to be five residues in length (Caporale et al., 1980). Human C3a-(73–77)-pentapeptide, Leu-Gly-Leu-Ala-Arg or **1**, is 10% as active as native human C3a in stimulating the release of histamine from human mononuclear leukocytes (Glovsky et al., 1979) and 0.2% as active as C3a in contracting isolated muscle strips from guinea pig ileum (Caporale et al., 1980). The present study has examined spasmogenic activity as measured by the ileal contraction assay in order to elucidate structural features of Leu-73, Gly-74, Leu-75, and Ala-76 that contribute to the interaction of active-site peptide **1** with the cellular C3a receptor. These results may be extrapolated to the interaction of native C3a with its cellular receptor.

Lipophilic Contributions of Leu-73 and Leu-75. The contractile activity of the active-site peptide **1** is decreased 40-fold by omission of Leu-73 (Caporale et al., 1980), 5-fold by replacement of Leu-73 by alanine, and at least 70-fold by replacement of Leu-75 by alanine. Thus, substitution of the isobutyl group of Leu-75 by a methyl group substantially decreases the apparent binding of the active-site analogue to the C3a receptor.

Substitution of Leu-73 by isoleucine, methionine, phenylalanine, or valine furnished analogues having similar smooth muscle activity to that of peptide **1**. Thus, replacement of the isobutyl group of Leu-73 by the larger benzyl group of phenylalanine, longer methylthioethyl chain of methionine, the shorter isopropyl group of valine, or the isomeric *sec*-butyl group of isoleucine has little effect on apparent receptor binding. Although a large lipophilic side chain is required at position 73 for high activity, the precise size or shape of this lipophilic side chain is not critical.

It is noteworthy that the presence of the benzyl group of phenylalanine or the 4-hydroxybenzyl group of tyrosine at position 73 actually increases the contractile activity to about 150% that of peptide **1**. Thus, this position can tolerate a side chain having significantly greater size and lipophilicity than provided by the naturally occurring leucyl residue.

In contrast, substitution of Leu-75 by methionine, phenylalanine, or valine gave analogues with little or no activity at the doses tested. Only Ile-75 analogue **14**, which is an isomer of peptide **1** having the isobutyl side chain at position 75 replaced by a *sec*-butyl side chain, showed modest (10%) activity. Since this analogue differs from peptide **1** by having one of the two outer methyl groups moved from the γ carbon to the β carbon, the presence of the second methyl group on the γ carbon of the Leu-75 side chain is evidently important for high contractile activity. The lipophilic contribution of Leu-73 to receptor binding is modest and sterically nonspecific while that of Leu-75 is substantial and sterically specific.

Orienting Contributions of Gly-74 and Ala-76. Substitution of one α hydrogen atom of Gly-74 by the methyl group of alanine or the hydroxymethyl group of serine produced modest changes in ileal activity. Ala-74 analogue **8** is the most active

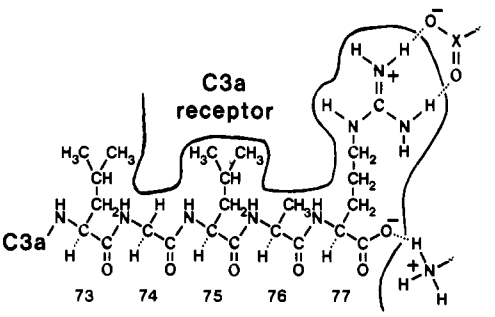

FIGURE 1: Conceptual model of the COOH-terminal active-site pentapeptide of C3a bound to its cellular receptor.

of the 23 pentapeptides described in this paper, exhibiting nearly 3 times the activity of peptide **1**. Even the Ser-74 analogue (**9**) was half as active as peptide **1**. Thus, a small side chain can be accommodated at position 74 with little effect on activity. In contrast, substitution of the methyl group of Ala-76 by either the hydrogen atom of glycine or the hydroxymethyl group of serine resulted in a substantial decrease in contractile activity.

The main-chain dihedral angles of alanine are conformationally much more restricted than those of glycine. Evidently, substitution of alanine for glycine at position 74 enhances the functional conformation(s) of peptide **1** at the receptor. Functional conformations of peptide **1** also appear to require the constraint of the methyl side chain at position 76, as indicated by the inactivity of the Gly-76 analogue (**22**) lacking this methyl group.

Replacement of Ala-76 by larger residues such as glutamine or glutamic acid resulted in little or no activity. The Gln-76 analogue (**20**) is only about 3% as active as peptide **1**, and the Glu-76 analogue **23** shows negligible binding to the C3a receptor. When present at 38 times the minimum active dose of peptide **1**, the latter failed to contract ileal strips or to desensitize them to subsequent contraction by C3a. These results suggest that the presence of a residue with a bulky side chain at either position 74 or 76 is not compatible with maintenance of substantial spasmogenic activity. The orienting contribution of Gly-74 to apparent receptor binding is neither conformationally nor sterically specific, while the contribution of Ala-76 is both conformationally and sterically specific.

Active Site of C3a. In a companion study,[1] we have defined the structural contributions of Arg-77 to the spasmogenic activity of a hexapeptide model of the COOH-terminal active site of C3a (Erickson et al., 1980, 1981). Essentially all of the structural features of this COOH-terminal arginyl residue examined were required for efficient contractile activity, and most were needed to achieve any demonstrable activity. The negatively charged carboxylate group of Arg-77 probably binds to a positively charged group of the receptor such as an ammonium or guanidinum group. The positively charged guanidinum group of Arg-77 probably binds in bidentate fashion to a negatively charged group of the receptor such as a carboxylate or phosphate group. The trimethylene moiety of the Arg-77 side chain serves as the most efficient spacer between the carboxylate and guanidinum groups. Finally, the chirality of Arg-77 must be L rather than D for any demonstrable binding.

A conceptual model for the active site of C3a anaphylatoxin is shown in Figure 1. The main contributions of large, lipophilic residues Leu-73 and Leu-75 are to bind to lipophilic sites of the cellular C3a receptor. The primary contributions of small, hydrophilic residues Gly-74 and Ala-76 are to position and to orient the side chains of Leu-73, Leu-75, and Arg-77

Table II: COOH-Terminal Sequences of C3a, C4a, and C5a

peptide	species	COOH-terminal sequence[a]
		73 77
C3a	man, pig, rat, mouse	-Leu-Gly-Leu-Ala-Arg-OH
		73 77
C4a	man	-Ala-Gly-Leu-Gln-Arg-OH
		73 77
C4a	cow	-Val-Gly-Leu-Ala-Arg-OH
		70 74
C5a	man	-Met-Gln-Leu-Gly-Arg-OH
		70 74
C5a	pig	-Ile-Gln-Leu-Gly-Arg-OH

[a] Residues different from C3a are underlined.

for effective receptor interaction. Residues Leu-73 and Gly-74 can be replaced efficiently by structurally similar residues. Since at very high doses even the peptide Leu-Ala-Arg induces the wheal and flare response (Caporale et al., 1980), they are not strictly required for receptor binding. In contrast, residues Leu-75 and Ala-76 are specifically required for efficient binding to the cellular C3a receptor.

The lipophilic contribution of Leu-75, orienting contribution of Ala-76, and ionic contributions of Arg-77 are each specific and quite important for efficient binding of active-site peptide **1** to the C3a receptor. The lipophilic contribution of Leu-73 and the orienting contribution of Gly-74 are less specific but quantitatively still important for receptor binding. Since longer synthetic peptides containing 8 or 13 residues from the COOH terminus of human C3a are about 12 and 30 times more active than pentapeptide **1**, respectively (Hugli & Erickson, 1977; Caporale et al., 1980), additional NH_2-terminal residues enhance apparent receptor binding.

On the basis of these studies, we can schematically view the cellular C3a receptor as containing a highly selective binding site that is complementary to the C3a active-site pentapeptide, as illustrated in Figure 1. Specifically, the C3a receptor probably contains lipophilic groups, a negatively charged bidentate group, and a positively charged group that are sterically positioned to favor binding to peptides ending in -Leu-Ala-Arg-OH.

As shown in Table II, COOH-terminal active-site pentapeptide **1** is common to the C3a anaphylatoxins from man (Hugli, 1975), pig (Corbin & Hugli, 1976), rat (Jacobs et al., 1978), and mouse (Domdey et al., 1982) but differs from the COOH-terminal pentapeptides of the C4a anaphylatoxins from man (Moon et al., 1981) and cow (Fothergill & Smith, 1982) and the C5a anaphylatoxins from man (Fernandez & Hugli, 1978) and pig (Gerard & Hugli, 1980). Human C4a binds to the same cellular receptor as C3a but exhibits only 1% of the spasmogenic activity of C3a (Gorski et al., 1979). This low level of activity is due to replacement of Leu-73 by alanine and Ala-76 by glutamine (Hugli et al., 1983). We expect that bovine C4a should be significantly more active than human C4a because its active-site pentapeptide is the Val-73 analogue (**5**), which is essentially as active as C3a active-site peptide **1** in the ileal contraction assay. The cellular C5a receptor, however, is not stimulated by factors that trigger degranulation through the C3a receptor (Fernandez & Hugli, 1976). None of the C3a active-site analogues examined in this study were able to desensitize the tissue to subsequent challenge by human C5a, and conversely, human C5a failed to inhibit the contractile activity of these C3a peptide analogues.

Acknowledgments

We thank Maria Pospichil, Laura Benedetto, and Marleen S. Kawahara for their excellent technical assistance.

Registry No. **1**, 66157-46-8; **2**, 76016-63-2; **3**, 76016-64-3; **4**, 88272-23-5; **5**, 88272-24-6; **6**, 76016-65-4; **7**, 75567-96-3; **8**, 88272-25-7; **9**, 88272-26-8; **10**, 88272-27-9; **11**, 88272-28-0; **12**, 88272-29-1; **13**, 88272-30-4; **14**, 88272-31-5; **15**, 88272-32-6; **16**, 76016-66-5; **17**, 88272-33-7; **18**, 75567-97-4; **19**, 88272-34-8; **20**, 86917-71-7; **21**, 88272-35-9; **22**, 88272-36-0; **23**, 88272-37-1; complement C3a, 80295-42-7.

References

Bokisch, V. A., & Müller-Eberhard, H. J. (1970) *J. Clin. Invest. 49*, 2427–2436.

Caporale, L. H., Tippett, P. H., Erickson, B. W., & Hugli, T. E. (1980) *J. Biol. Chem. 255*, 10758–10763.

Cochrane, C. G., & Müller-Eberhard, H. J. (1968) *J. Exp. Med. 127*, 371–386.

Corbin, N. C., & Hugli, T. E. (1976) *J. Immunol. 117*, 990–995.

Domdey, H., Wiebauer, K., Kazmaier, M., Muller, V., Odink, K., & Fey, G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7619–7623.

Erickson, B. W., & Merrifield, R. B. (1976) *Proteins (3rd Ed.) 2*, 255–527.

Erickson, B. W., Caporale, L. H., Fok, K. F., Unson, C. G., & Hugli, T. E. (1980) *J. Immunol. 104*, 1519.

Erickson, B. W, Fok, K. F., Khan, S. A., Lukas, T. J., Molinar, R. R., Munoz, H., Prystowsky, M. B., Unson, C. G., Volk-Weiss, J., & Hugli, T. E. (1981) in *Peptides: Synthesis, Structure, Function* (Rich, D. H., & Gross, E., Eds.) pp 525–534, Pierce Chemical, Rockford, IL.

Fernandez, H., & Hugli, T. E. (1976) *J. Immunol. 117*, 1688–1694.

Fernandez, H. N., & Hugli, T. E. (1978) *J. Biol. Chem. 253*, 6955–6964.

Fothergill, J. E., & Smith, M. A. (1982) *Mol. Immunol. 19*, 1370.

Gerard, C., & Hugli, T. E. (1980) *J. Biol. Chem. 255*, 4710–4715.

Glovsky, M. M., Hugli, T. E., Ishizaka, T., Lichtenstein, L. M., & Erickson, B. W. (1979) *J. Clin. Invest. 64*, 804–811.

Gorski, J. P., Hugli, T. E., & Müller-Eberhard, H. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5299–5302.

Huber, R., Scholze, H., Paques, E. P., & Deisenhofer, J. (1980) *Hoppe-Seyler's Z. Physiol. Chem. 361*, 1389–1399.

Hugli, T. E. (1975) *J. Biol. Chem. 250*, 8293–8301.

Hugli, T. E., & Erickson, B. W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1826-1830.

Hugli, T. E., & Müller-Eberhard, H. J. (1978) *Adv. Immunol. 26*, 1–53.

Hugli, T. E., Kawahara, M. S., Unson, C. G., Molinar-Rode, R., & Erickson, B. W. (1983) *Mol. Immunol. 20*, 637–645.

Jacobs, J. W., Rubin, J. S., Hugli, T. E., Bogardt, R. A., Mariz, I. K., Daniels, J. S., Daughaday, W. H., & Bradshaw, R. A. (1978) *Biochemistry 17*, 5031–5037.

Khan, S. A., & Erickson, B. W. (1981) *J. Am. Chem. Soc. 103*, 7374–7376.

Khan, S. A., & Erickson, B. W. (1982) *J. Biol. Chem. 257*, 11864–11867.

Law, S. K., Lichtenberg, N. A., & Levine, R. P. (1979) *J. Immunol. 123*, 1388–1399.

Lepow, I. H., Wilhus-Krelschen, K., Patrick, R. A., & Rosen, F. S. (1970) *Am. J. Pathol. 61*, 13–23.

Moon, K. E., Gorski, J. P., & Hugli, T. E. (1981) *J. Biol. Chem. 256*, 8685–8692.

Morgan, E. L., Weigle, W. O., & Hugli, T. E. (1982) *J. Exp. Med. 155*, 1412–1426.

Tack, B. F., Harrison, R. A., Janatova, J., Thomas, M. L., & Prahl, J. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5764-5768.

Unson, C. G., Erickson, B. W., & Hugli, T. E. (1979) in *Peptides: Structure and Biological Function* (Gross, E., & Meinhofer, J., Eds.) pp 459-462, Pierce Chemical, Rockford, IL.

Weigle, W. O., Morgan, E. L., Goodman, M. G., Chenoweth, D. C., & Hugli, T. E. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 3099-3103.

Azo Protein Analogues: Synthesis and Characterization of Arsanilazo and Sulfanilazo Derivatives of Tyrosine and Histidine†

Gary J. Pielak,‡ Mickey S. Urdea,§ Kozo Igi,‖ and J. Ivan Legg*

ABSTRACT: The synthesis, purification, and characterization of the products obtained upon reaction of N^{α}-acetyl-L-histidine and *N*-acetyl-L-tyrosine with either diazotized arsanilic or sulfanilic acid were undertaken. The products previously reported to be pure monoazo- and bisazo-L-tyrosine and -L-histidine [Tabachnick, M., & Sobotka, H. (1959) *J. Biol. Chem. 234*, 1726] are shown to consist of a number of chromophoric species. The visible spectra described here differ substantially from the earlier report. To date, the use of the visible spectral properties of these azo protein analogues to determine the extent of histidine and tyrosine modification of proteins with diazotized arsanilic or sulfanilic acid has been in error. From the model studies reported here, it is now possible to characterize the corresponding azo proteins. It is also possible to distinguish spectrally between the C-2 and C-4 azo-coupled products of the histidine imidazole. Characterization of azo proteins is essential to metal-azo protein incorporation studies under investigation.

Simple and systematic methods for the incorporation of substitution-inert metal ions into proteins are under investigation in this laboratory. A particularly promising method involves the production of an azo dye chelating agent on the protein with a diazonium salt followed by incorporation of Co(III). We have successfully applied this methodology to carboxypeptidase A azo coupled[1] at tyrosine-248 (Urdea & Legg, 1979a,b) and have reported preliminary results for several other proteins (Urdea et al., 1979). As a first step toward making this method generally applicable, we have undertaken a reinvestigation of protein azo coupling.

Typically, determination of the extent and specificity of diazonium salt incorporation has been based on the visible spectral properties (band position and molar absorptivities) of the azo proteins as compared to simple azo amino acids (Tabachnick & Sobotka, 1960).[2] We have found that for characterization of proteins modified with either diazotized arsanilic or sulfanilic acid, this technique is highly erroneous since the compounds originally reported to be homogeneous are mixtures of chromophoric species. These compounds have now been purified and characterized, and it is possible to distinguish spectrally between C-2 and C-4 azo-coupled histidine.

Experimental Procedures

Preparation of Arsanilazo Derivatives of N-Acetyl-L-tyrosine. The method of synthesis was similar to that described by Tabachnick & Sobotka (1959). Arsanilic acid (Aldrich Chemicals, 0.651 g) was dissolved in 50.0 mL of 0.30 N HCl at 0 °C. Sodium nitrite (0.311 g) and sodium bromide (0.062 g) were added, and the solution was stirred for 30 min. The diazonium salt was added over a 30-min period to 25.0 mL of 0.120 M *N*-acetyl-L-tyrosine (Vega) and 0.01 M borate, pH 9.0 at 0 °C, and 1 N NaOH was added as necessary to keep the pH between 9.0 and 9.5. The reaction was stirred for an additional 3.5 h, turning first yellow then yellowish brown.

Isolation of N-Acetylmono(arsanilazo)-L-tyrosine (NAMAAT).[3] The above solution was acidified to pH 1.8 with 1 N HCl. The yellow-orange precipitate was filtered and washed with a small portion of cold water. The product (ca. 150 mg) was dissolved in 1 mL of the 50/50 ISTEA solvent

† From the Department of Chemistry and the Program in Biochemistry and Biophysics, Washington State University, Pullman, Washington 99164. *Received March 28, 1983; revised manuscript received September 19, 1983.* This study was supported by the National Institutes of Health (GM 23081). The Nicolet 200-MHz spectrometer was acquired with the assistance of the Boeing Co. M.S.U. was supported in part by a C. Glenn King Fellowship, Washington State University. Studies reported were submitted by G.J.P. in partial fulfillment of the requirements for the Ph.D. degree of Washington State University.

‡ Present address: Department of Biochemistry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5.

§ Present address: Chiron Corp., Emeryville, CA 94608.

‖ Present address: Callery Chemical Co., Callery, PA 16024.

[1] The terms "diazotization" and "diazotized" have been used in previous work to indicate both the formation and coupling of diazonium salts. However, in this paper, in accordance with current usage, these terms refer only to diazonium salts while "azo coupling" and "coupled" refer to the reaction of diazonium salts.

[2] The equations of Tabachnick & Sobotka (1960) later appeared (and were cited) in Riordan & Vallee (1972).

[3] Abbreviations: NAMAAT, *N*-acetylmono(arsanilazo)-L-tyrosine; NABAAT, *N*-acetylbis(arsanilazo)-L-tyrosine; NAMAAH(C-2), N^{α}-acetyl-2-mono(arsanilazo)-L-histidine; NAMAAH(C-4), N^{α}-acetyl-4-mono(arsanilazo)-L-histidine; NABAAH, N^{α}-acetylbis(arsanilazo)-L-histidine; NAMSAT, *N*-acetylmono(sulfanilazo)-L-tyrosine; NABSAT, *N*-acetylbis(sulfanilazo)-L-tyrosine; NAMSAH(C-2), N^{α}-acetyl-2-mono(sulfanilazo)-L-histidine; NAMSAH(C-4), N^{α}-acetyl-4-mono(sulfanilazo)-L-histidine; NABSAH, N^{α}-acetylbis(sulfanilazo)-L-histidine; ISTEA, distilled 2-propanol and triethylamine bicarbonate, pH 9.5; ^{1}H NMR, proton nuclear magnetic resonance; TLC, thin-layer chromatography.

0006-2960/84/0423-0589$01.50/0