Synthetic Analogs of the Hypothalamic Luteinizing Hormone Releasing Factor with Increased Agonist or Antagonist Properties†

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ABSTRACT: Peptide analogs of the hypothalamic hormone, luteinizing hormone releasing factor (LRF, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), altered at the 6 position were synthesized by the solid-phase method and tested *in vitro* and *in vivo* for the ability to induce secretion of the pituitary hormone, luteinizing hormone. One of these analogs, [D-Ala^6]LRF, exhibits 350–450% of the potency of the parent hormone. The high activity of this analog is interpreted in terms of conformation and a model is proposed to explain this result. The D-alanine modification was incorporated into a known competitive inhibitor (des-His^2-LRF); the resulting compound, des-His^2-[D-Ala^6]LRF, is 300% more potent than des-His^2-LRF in its ability to antagonize the action of LRF.

The hypothalamic hormone LRF (luteinizing hormone releasing factor, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) stimulates the secretion of the pituitary hormone(s) which regulate ovulation (Matsuo *et al.*, 1971; Monahan *et al.*, 1971; Amoss *et al.*, 1972a). Part of its mechanism of action involves the interaction of LRF with pituitary cell receptors, and structure–activity relationships of analogs of LRF can provide insight into this interaction. Traditionally, the study of structure–activity relationships in peptide hormones has been the study of the functionality of the side chains of the amino acids of the hormones. However, the role of conformation has received considerable attention recently, and it has become necessary to correlate both conformation and structure–activity relationships. To this end, three series of LRF analogs have been synthesized involving substitution of the amino acids of the decapeptide with glycine, alanine, or D-alanine (Monahan *et al.*, 1972a,b, 1973). The glycine- and alanine-substituted analogs allow evaluation of the functional properties of the individual side chains; D-alanine analogs provide comparative information on the backbone structure of the peptide.

One of the primary aims of these studies is to obtain information which will allow the design of efficient antagonists of LRF as a new approach to fertility regulation (Vale *et al.*, 1972b). Indeed, [Gly^2]LRF and des-His^2-LRF have already been found to be competitive inhibitors of LRF (Monahan *et al.*, 1973, 1972b; Vale *et al.*, 1972b). This paper describes analogs of LRF among which one, [D-Ala^6]LRF, has greater potency than LRF, and another, des-His^2-[D-Ala^6]LRF, is an inhibitor of higher potency than des-His^2-LRF.

Experimental Procedures

Each synthesis was carried out on a benzhydrylamine resin (1 g; 0.4 mequiv of amine/g of resin) as described previously (Merrifield, 1963; Pietta and Marshall, 1970; Monahan *et al.*, 1972b; Monahan and Rivier, 1972). Completed resin–peptides were cleaved in *ca.* 10 ml of anhydrous HF containing 25% anisole for 1 hr at 0°. HF was removed under vacuum and the resulting mixture was extracted with 50 ml of ethyl acetate and filtered to remove anisole. The peptide was then washed from the resin with acetic acid and water. The ethyl acetate extract was backwashed with water and the combined aqueous layers evaporated. The residual oil was either lyophilized or applied directly to a partition column of Sephadex G-25 (fine) which had been equilibrated with the lower and then the upper phase of the solvent system (system A) *n*-butyl alcohol–acetic acid–water (4:1:5).[1] Elution with the upper phase yielded a material which was judged pure or submitted to further chromatographic purification on a column prepared from Sephadex G-25 (fine) which had been equilibrated with 0.5 N acetic acid (system B). Peptide fractions located by ultraviolet (uv) (280 nm) absorption were pooled and lyophilized from 0.5 N acetic acid to obtain white powders in all cases.

Purification details follow and include solvent system (s) used, column dimensions, R_F values ($R_F = V_v/V_e$, where V_v is the column void volume and V_e is the elution volume of the peptide), and yield based on starting substitution: [Ala^6]LRF, system A, 1.6 cm × 190 cm column, R_F 0.28–0.24, 85 mg (17% yield); [D-Ala^6]LRF, system A, 1.6 cm × 190 cm column, R_F 0.33–0.30, followed by system B, 2 cm × 100 cm column, R_F 0.4–0.36, 120 mg (24% yield); [Val^6]LRF, system A, 2 cm × 100 cm column, R_F 0.24–0.22, followed by system B, identical column, R_F 0.40–0.36, 58 mg (11% yield); [D-Val^6]LRF, system A, 2 cm × 100 cm column, R_F 0.42–0.38, followed by system B, identical column, R_F 0.43–0.40, 95 mg (19% yield); [Pro^6]LRF, system A, 2 cm × 100 cm column, R_F 0.19–0.17,

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[1] The partition column chromatographic technique of system A has been used in the purification of analogs of LRF substituted with glycine and alanine. Analogs in which the side chains of His, Trp, Tyr, Leu, and Pro are substituted yield significantly different R_F values than LRF, implying that the side chains of these residues contribute strongly to the partition coefficient of LRF in system A. Therefore, it is likely that failure sequence contaminants involving these residues are separated from the main fraction containing the decapeptide of interest. Indeed, we have observed distinct separation of several failure sequence peptides during the purification of LRF and several analogs. It is of significance that many of these materials, while separable by partition chromatography, give identical R_F values to parent compounds on thin layer chromatography(for details, see Yamashiro, 1964).

TABLE I: Physical Characteristics of LRF Analogs.

Analog	$[\alpha]_D$[a] (deg)	R_F(I)[b]	R_F(II)	R_F(III)	R_F(IV)	R_F(V)	R_F(VI)	R_F(VII)
[Ala^6]LRF	-59.9 ± 0.3, c 1.086	0.27	0.68	0.48	0.71[c]	0.67	0.30	0.41
[D-Ala^6]LRF	-40.5 ± 0.4, c 0.746	0.27	0.68	0.46	0.71	0.68	0.31	0.42
[Val^6]LRF	-61.1 ± 0.1, c 1.019	0.31	0.71	0.50	0.71	0.72	0.36	0.44
[D-Val^6]LRF	-38.9 ± 0.2, c 0.957	0.30	0.70	0.50	0.74	0.72	0.35	0.45
[Pro^6]LRF	-81.7 ± 0.2, c 1.118	0.26	0.68	0.45	0.69	0.70	0.30	0.42
[D-Pro^6]LRF	-19.5 ± 0.1, c 1.038	0.28[d]	0.70	0.51	0.74	0.71	0.32	0.44
Des-His^2-[D-Ala^6]LRF	-34.8 ± 0.1, c 1.050	0.30	0.79	0.52	0.80	0.73	0.47	0.45

[a] Rotations measured (ten determinations) in 1% acetic acid on a Perkin-Elmer Model 141 polarimeter. [b] R_F values for 20–30-μg load on Eastman 6060 precoated silica gel plates in the following solvent systems: I, n-BuOH–i-PrOH–1 N NH_3–EtOAc (1:1:5:1); II, i-PrOH–1 N AcOH (2:1); III, 0.1% AcOH–n-BuOH–Pyr (11:5:3); IV, EtOAc–Pyr–AcOH–H_2O (5:5:1:3); V, i-PrOH–1 N NH_3 (2:1); VI, n-BuOH–AcOH–H_2O (4:1:5); VII, isoamyl alcohol–Pyr–H_2O (7:7:6). Plates were examined for fluorescent material under uv light, exposed to iodine vapor, treated with ninhydrin reagent (all compounds negative), and then sprayed with Pauly reagent. [c] Minor component at solvent front. [d] Minor component at R_F 0.31.

TABLE II: Amino Acid Ratios of LRF Analogs.

Analog	Glu	His	Trp	Ser	Tyr	X[6]	Leu	Arg	Pro	Gly	NH_3
[Ala^6]LRF[a]	1.00	0.88	0.94	0.93	0.97	0.99	1.00	0.96	1.03	1.09	1.30
[D-Ala^6]LRF[a]	1.01	0.96	1.19	0.87	0.98	0.98	1.00	1.00	0.99	0.99	0.93
[Val^6]LRF[b]	1.04	0.96	1.03	0.84	0.94	0.99	1.00	0.97	0.98	0.98	1.09
[D-Val^6]LRF[b]	1.01	0.95	1.03	0.82	0.95	1.00	1.00	0.97	0.99	1.00	1.15
[Pro^6]LRF[a]	0.99	0.86	1.03	0.96	0.99		1.00	0.95	2.13	1.02	0.79
[D-Pro^6]LRF[a]	0.96	0.88	1.14	0.94	0.97		1.00	0.97	2.06	0.99	0.94
Des-His^2-[D-Ala^6]LRF[a]	1.04		0.63	0.89	0.99	1.00	1.00	0.99	1.08	1.07	0.87

[a] In order to obtain amino acid ratios, peptides were hydrolyzed in 6 N HCl containing 0.5% thioglycolic acid in sealed tubes (20 μ pressure, 110°, 20 hr). The resulting hydrolysates were subjected to amino acid analysis on a Model 119 Beckman-Spinco amino acid analyzer. Ratios are uncorrected and are normalized to leucine. [b] Amino acid ratios obtained as above except that hydrolyses were carried out for 72 hr to ensure hydrolysis of Val-Leu sequences.

followed by system B, identical column, R_F 0.43–0.36, 100 mg (20% yield); [D-Pro^6]LRF, system A, 2 cm × 100 cm column, R_F 0.31–0.28, 73 mg (14% yield); des-His^2-[D-Ala^6]-LRF, system A, 2 cm × 100 cm column, R_F 0.52–0.44, followed by system B, identical column, R_F 0.36–0.32, 112 mg (25% yield).

All compounds were submitted to thin-layer chromatography (tlc) in the seven solvent systems indicated (20–30-μg load on Eastman 6060 silica gel plates): system I, n-butyl alcohol–isopropyl alcohol–1 N NH_3–ethyl acetate (1:1:5:1); II, isopropyl alcohol–1 N acetic acid (2:1); III, 0.1% acetic acid–n-butyl alcohol–pyridine (11:5:3); IV, ethyl acetate–pyridine–acetic acid–water (5:5:1:3); V, isopropyl alcohol–1 N NH_3 (2:1); VI, n-butyl alcohol–acetic acid–water (4:1:5); VII, isoamyl alcohol–pyridine–water (7:7:6). Plates were examined under uv light, treated with iodine vapor, and sprayed with ninhydrin and then Pauly reagent. All compounds are homogeneous on these systems except [Ala^6]LRF and [D-Pro^6]LRF as noted in the footnotes to Table I.

Rotations at 589 nm were measured in 1% acetic acid on a Perkin-Elmer Model 141 polarimeter (Table I). In order to obtain amino acid ratios, peptides were hydrolyzed at 110° for 20 hr in 6 N HCl containing 0.5% thioglycolic acid at 20 μ pressure. Compounds containing valine were hydrolyzed for 72 hr under the same conditions. The resulting hydrolysates were submitted to amino acid analysis on a Model 119 Beckman-Spinco amino acid analyzer (see Table II).

Agonist activity of each synthetic analog was compared to the LRF standard by measurement *in vivo* of the elevation in plasma luteinizing hormone (LH) 15 min after an iv injection of the test compounds in chronically ovariectomized rats given 50 μg of 17β-estradiol on each of the 3 days prior to the assay. Plasma LH levels were measured by a solid-phase radioimmunoassay and are evaluated in terms of the NIAMD-RLH-RP-1 standard. Strict four-point bioassays were performed on the data after covariance adjustments relating the pre- and postinjection LH concentrations. Indices of precision ($\lambda = s/m$) of the potency estimates ranged from 0.15 to 0.3 on samples exhibiting biological activity (Amoss *et al.*, 1972b).

Measurement by double antibody radioimmunoassay of the LH released from enzymatically dispersed rat pituitary cell cultures is the basis of an *in vitro* assay for the estimation of agonist biological activity of the various analogs of LRF (Vale *et al.*, 1972a) as well as an antagonist activity to LRF. Dose–response curves were obtained using multiple doses of LRF standard and analogs being tested; the exact experimental design for the *in vitro* testing of antagonists to LRF is described in Vale *et al.* (1972b).

Results and Discussion

The analog [Ala^6]LRF exhibits *ca.* 4% of the potency of LRF both *in vivo* and *in vitro*, while [D-Ala^6]LRF has 350–

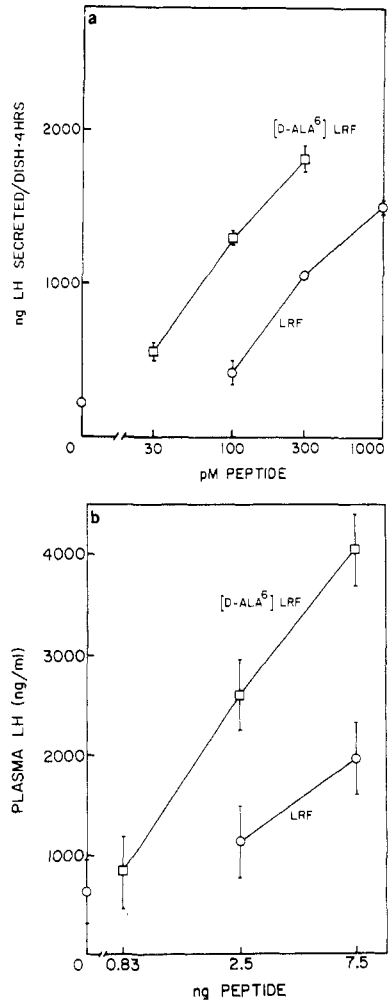

FIGURE 1: (a) The effect of LRF concentration (circles) and [D-Ala^6]LRF concentration (squares) on the secretion of LH by pituitary cell cultures. (b) The effect of LRF and [D-Ala^6]LRF on the level of LH concentration in the plasma of ovariectomized 17β-estradiol pretreated rats.

450% of the activity of LRF (Table III and Figures 1a and b).[2] It is unlikely that differences in clearance rates of the peptides could account for the differences in biological potencies, since potencies determined *in vitro* are in agreement with *in vivo* results. Also, possible differential rates of serum inactivation can be ruled out as an explanation for the relative potencies of these peptides, since the *in vitro* assay is carried out in the absence of serum. The effect of the substitution of the sixth residue (glycine) with D- or L-alanine can best be interpreted as a change in the conformation of the molecule at the binding site (the methyl group of the D-alanine side chain could not likely be implicated in a process involving functionality). The increased biological activity of [D-Ala^6]LRF can be explained as the result of a restriction in the conformational degrees of freedom of LRF when D-alanine is substituted for glycine. This restriction could lead to the stabilization of a conformation favorable for binding, and hence activity at the receptor.

TABLE III: Agonist Activities of LRF Analogs.

Analog	% Potency (*In Vivo*)[a]	% Potency (*In Vitro*)[b]
[Ala^6]LRF	4.3 (1.8–24)	4
[D-Ala^6]LRF	357 (150–909)	474 (404–563) 415 (322–541)
[Val^6]LRF	<0.1	0.01
[D-Val^6]LRF	32 (14–76)	30
[Pro^6]LRF	1.5 (1.0–4.3)	3
[D-Pro^6]LRF	3.3 (0.9–7.1)	10
Des-His^2-[D-Ala^6]LRF[c]	<0.01	<0.01

[a] Based on ability to release luteinizing hormone in ovariectomized, steroid-treated rats using LRF standard. Confidence limits are shown in parentheses. LRF standard is taken as 100%. [b] Based on ability to induce secretion of luteinizing hormone in primary cultures of dispersed rat pituitary cells. LRF standard is taken as 100%. [c] Des-His^2-[D-Ala^6]LRF antagonizes the action of LRF *in vitro*.

There are probably a large number of conformational states which could explain the difference in potency between [Ala^6]-LRF and [D-Ala^6]LRF. These include the helices (Pauling and Corey, 1950; Pauling *et al.*, 1951), the type I and type II β bends (Geddes *et al.*, 1968), the γ turn (Neméthy and Printz, 1972), and the extended form. The helical structures can be ruled out immediately, since the presence of a D-amino acid would disrupt such a structure. The remaining defined forms are possible, but correlative evidence in the literature indicates that the β-II type bend (Figure 2) best explains the data presented here. Geddes (Geddes *et al.*, 1968) proposed the β-II type bend and the β-I type bend (Figure 2) in order to explain X-ray diffraction and infrared dichroism measurements on Chrysopa silk fibers. They propose that the polypeptide chains of these silk fibers form antiparallel pleated sheets by incorporation of a β-type bend at every eighth amino acid residue (glycine). These bends are defined in the two forms shown in Figure 2, and each involves a ten-membered hydrogen-bonded ring structure. Venkatachalam (1968) demonstrated that, because of steric constraints, a hydrogen bond between the carbonyl group of residue i (Figure 2) and the amide group of residue $i + 3$ can be formed in a β-type bend only if glycine or D-alanine occurs in position $i + 2$. Némethy and Printz (1972) performed semiempirical conformational energy calculations on various types of turns in peptide sequences and found that when D-alanine is substituted for L-alanine in residue $i + 2$, the β-II type bend is stabilized by 4.7 kcal and both the β-I and γ bends are destabilized by this substitution. Thus, there is justification for the hypothesis that [D-Ala^6]LRF may exist more extensively in a conformation incorporating a β-II type bend than [Ala^6]LRF at the binding site.

There is correlative evidence consistent with the hypothesis that LRF may also exist, at least partially, with a β bend. Lewis and coworkers (1971) performed a statistical analysis on the probability of occurrence of amino acids involved in β-type bends in three proteins whose tertiary structures are known (hen egg-white lysozyme, bovine ribonuclease S, and bovine α-chymotrypsin). They found that serine occurs 55 times in these proteins, and of these, it is in a β-type bend 28 times and in the i position of the bend 11 of the 28 times.

[2] The low level of biological activity observed for [Ala^6]LRF could be due to a very small amount of racemization during the coupling of *tert*-butoxycarbonyl-L-alanine with dicyclohexylcarbodiimide during the synthesis. Only 1% contamination with D-alanine could account for 4% potency in this preparation of [Ala^6]LRF. The important point is that [Ala^6]LRF is much less potent than LRF or [D-Ala^6]LRF, and that partial racemization of L-alanine would serve only to *decrease* the observed difference in potency of these two preparations.

Similarly, tyrosine occurs 13 times, six located in β-type bends, and two of the six times in the $i + 1$ position. Glycine occurs 36 times, 21 located in β bends, and of the 21 it is in the $i + 2$ position of the bend 11 times. Leucine occurs less predominantly in β bends, occurring in these proteins 27 times, five of these in β bends, and in two of the five times it is in the $i + 3$ position. Furthermore, Crawford *et al.* (1973) have analyzed seven globular proteins and have established that glycine occurs in 15 β-II type bends and is in the $i + 2$ position of these bends 10 of the 15 times. These data support the proposal that the LRF sequence, Ser-Tyr-Gly-Leu-, may form a stable β-type bend with serine in the i position.

These calculations and the difference in biological potency between [Ala^6]LRF and [D-Ala^6]LRF are consistent with the proposal that the receptor recognizes LRF more effectively when the hormone is in the β-II conformation as shown in Figure 1. Biological data on other LRF analogs altered at the 6 position are consistent with this hypothesis (Table III). The analogs having L residues in the sixth position are lower in potency (Table III) than those having D-amino acids in that position. Analogs having D-amino acids with side chains of large bulk in this position (Table III) are less potent than [D-Ala^6]LRF; this may be explained by steric considerations (from intramolecular interactions and possible interactions at the binding site). It is possible, however, that variants of the γ turn or the two types of β bends could also explain the difference in potency between [D-Ala^6]LRF and [Ala^6]LRF. Also, a conformational alteration in one of the many extended forms could also account for this difference.

Molecular models do not give rigorously accurate representations of peptide conformation. However, examination of a Corey–Pauling–Koltun (CPK) molecular model of the LRF decapeptide in which the β-II turn is incorporated indicates that further structural features of LRF may be predicted. For instance, a second hydrogen-bonded ring structure seems likely in LRF. This second ring structure involves the carboxyl function of leucine (Leu^7) and the α-amide proton of serine (Ser^4) which would tend to stabilize the β-II bend.

It can be postulated that any modification in the LRF structure which results in increased specific activity of an LRF analog ought to be reflected in increased antagonist properties of LRF inhibitors incorporating that modification. Since LRF and [D-Ala^6]LRF exhibit parallel dose–response curves (Figure 1b) with similar response maxima (not shown), it is logical to assume that the increased specific activity of [D-Ala^6]LRF is due to increased binding affinity for the pituitary receptor sites. In addition, des-His^2-[D-Ala^6]LRF exhibits equivalent antagonism to LRF at one-third the dose required of des-His^2-LRF, also indicating increased binding affinity.

Publications from laboratories other than ours describe LRF analogs which are pertinent to the work presented here. Chang and collaborators (1972) present data on LRF analogs substituted at the 8 position (Arg) and speculate that the side chains of tryptophan and tyrosine exist in "parallel planarity" requiring a folding (unspecified) of the peptide to accomplish this interaction. A β-II type bend in LRF would exclude the possibility of the aromatic side chains of tyrosine (Tyr^5) and tryptophan (Trp^3) being close enough for such an interaction. However, tyrosine and tryptophan are placed on the same side of the molecule using the β-II-type bend conformation. If one of the faces of the receptor is a hydrophobic region in the membrane, then the positioning of these two aromatic residues could account for the increased ability of [D-Ala^6]LRF to bind at the site of action.

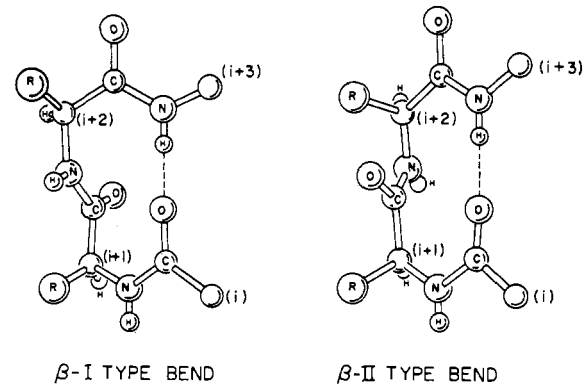

FIGURE 2: Two β-type bends proposed by Geddes *et al.* (1968). Serine occupies the i position (α carbons indicated), tyrosine the $i + 1$, glycine the $i + 2$, and leucine the $i + 3$ position of the β-II type bend proposed for LRF. Drawing adapted from Dickerson *et al.* (1971).

Fujino and coworkers (1972) have reported that des-Gly-NH$_2^{10}$-[Pro^9-ethylamide]LRF has five times the potency of LRF. They suggest that the total chain length of the hormone is important for binding. Obviously, conformational effects could be responsible for the increased potency (binding affinity) of this compound as in [D-Ala^6]LRF. Unfortunately, data regarding this modification of the tenth residue in LRF are not sufficient to draw a conclusion involving conformation.

In conclusion, the results presented here are best explained by proposing that the biological potency of LRF analogs can be related to conformational factors since the pituitary cell receptor has greater affinity for [D-Ala^6]LRF than for the parent hormone LRF. Thus, it is probable that the pituitary cell receptor prefers that LRF be in a specific conformational state which may contain a β-II type bend, a conformation which is stabilized in [D-Ala^6]LRF. Furthermore, modifications affecting binding which lead to increased potency in LRF analogs also lead to increased potency of competitive inhibitors of LRF to inhibit the agonist action of LRF.

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References

Amoss, M., Blackwell, R., and Guillemin, R. J. (1972a), *Clin. Endocrinol. Metab. 34*, 434.

Amoss, M., Rivier, J., and Guillemin, R. (1972b), *J. Clin. Endocrinol. Metab. 35*, 175.

Chang, J. K., Williams, R. H., Humphries, A. J., Johansson, N., Folkers, K., and Bowers, C. (1972), *Biochem. Biophys. Res. Commun. 47*, 727.

Crawford, J. L., Lipscomb, W. N., and Schellman, C. G. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 538.

Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O., Samson, L., Cooper, A., and Margoliash, E. (1971), *J. Biol. Chem. 246*, 1511.

Fujino, M., Kobayashi, S., Obayashi, M., Fukuda, T., Shinagawa, S., Yamazaki, I., Nakayama, R., White, W. F.,

and Rippel, R. H. (1972), *Biochem. Biophys. Res. Commun. 49*, 863.
Geddes, A. J., Parker, K. D., Atkins, E. D. T., and Beighton, E. (1968), *J. Mol. Biol. 32*, 343.
Lewis, P. N., Momany, F. A., and Scheraga, H. A. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2293.
Matsuo, H., Baba, Y., Nair, R. M. G., Arimura, A., and Schally, A. V. (1971), *Biochem. Biophys. Res. Commun. 43*, 1334.
Merrifield, R. B. (1963), *J. Amer. Chem. Soc. 85*, 2149.
Monahan, M., Rivier, J., Burgus, R., Amoss, M., Blackwell, R., Vale, W., and Guillemin, R. (1971), *C. R. Acad. Sci. 273*, 508.
Monahan, M., Rivier, J., Vale, W., Ling, N., Grant, G., Amoss, M., Guillemin, R., Burgus, R., Nicolaides, E., and Rebstock, M. (1972a), *Proc. 3rd Amer. Peptide Symp.*, 601.
Monahan, M. W., and Rivier, J. (1972), *Biochem. Biophys. Res. Commun. 48*, 1100.
Monahan, M. W., Rivier, J., Vale, W., Guillemin, R., and Burgus, R. (1972b), *Biochem. Biophys. Res. Commun. 47*, 511.
Monahan, M. W., Vale, W., Rivier, C., Grant, G., and Guillemin, R. (1973), Abstracts, The Endocrine Society, June.
Némethy, G., and Printz, M. P. (1972), *Macromolecules 5*, 755.
Pauling, L., and Corey, R. B. (1950), *J. Amer. Chem. Soc. 72*, 5349.
Pauling, L., Corey, R. B., and Branson, H. R. (1951), *Proc. Nat. Acad. Sci. U. S. 37*, 205.
Pietta, P. G., and Marshall, G. R. (1970), *Chem. Commun.*, 650.
Vale, W., Grant, G., Amoss, M., Blackwell, R., and Guillemin, R. (1972a), *Endocrinology 91*, 562.
Vale, W., Grant, G., Rivier, J., Monahan, M., Amoss, M., Blackwell, R., Burgus, R., and Guillemin, R. (1972b), *Science 176*, 933.
Venkatachalam, C. M. (1968), *Biopolymers 6*, 1425.
Yamashiro, D. (1964), *Nature (London) 201*, 76.

Structure of a λ-Type Bence-Jones Protein at 3.5-Å Resolution†

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ABSTRACT: An electron density map at 3.5-Å resolution has been calculated for a human λ-type Bence-Jones dimer. Organic mercurials, such as *o*-chloromercuriphenol, *p*-hydroxymercuribenzenesulfonate, methylmercuric chloride, and 2-ethylmercurithiosalicylate, were employed to prepare noncovalent isomorphous derivatives. A covalent S–Hg–S derivative, in which a mercury atom was inserted between the sulfur atoms in the interchain disulfide bond in the dimer, was also used to determine the phase angles for the native protein. The dimer is represented by two structural units or "modules." The larger module contains the N-terminal 111 amino acid residues of both monomers and the smaller module consists of the C-terminal 105 residues of the two chains. The amino and carboxyl regions ("domains") of each monomer are connected by an extended chain (the "switch" region). Although the three-dimensional structures of the two monomers are not equivalent, local pseudotwofold axes are found within each module. The axis between the amino domains intersects the axis between the carboxyl domains at an angle of 120°. The amino and carboxyl domains have structural similarities which support the hypothesis of a common ancestral protein. The basic structure of each domain consists of two layers. One layer contains three antiparallel segments of polypeptide chain; the second layer is composed of four segments. An intrachain disulfide bond bridges the two layers in the center of each domain. The three-chain layers in the smaller module (carboxyl domains) supply the outside surface; the four-chain layers provide close contacts at the interface between the two monomers. In an amino domain, the separation of the polypeptide chain into layers is less distinct than in a carboxyl domain. Contrary to expectations, the four-chain layers provide the outer surface of the larger module; the three-chain layers face each other to form a cavity, shaped like a truncated cone 15 Å across at the mouth and 10 Å deep. This cavity, which has many properties attributed to an antigen-binding site of an antibody, is lined by residues from the three "hypervariable" regions of the amino domains. The large side chains of residues 34 and 52 in both monomers protrude into the cavity. The floor of the cavity is composed of an array of four aromatic side chains, two from each monomer. The spatial relations between the amino and carboxyl domains are strikingly different in the two monomers. The evidence suggests that one of the light chains simulates the heavy chain in an antigen-binding (Fab) fragment.

Immunoglobulins are proteins with specific antibody activity or structural features closely resembling those of antibodies. IgG immunoglobulins from myelomatous or normal sera consist of two light (mol wt = 22,000–23,000) and two heavy chains (mol wt = 50,000–55,000). Free light chains excreted into the urine in patients with multiple myeloma are called Bence-Jones proteins, the presence of which can be used as a diagnostic test for the disease (Edelman and Gally, 1962).

We previously reported crystallographic studies of an unusual serum IgG1 immunoglobulin and the urinary λ-type Bence-Jones protein from a patient (Mcg) with multiple myeloma and amyloidosis (Schiffer *et al.*, 1970; Edmundson *et al.*, 1970, 1971, 1972). These proteins were originally isolated and characterized by Deutsch and his colleagues, with whom we are collaborating (Deutsch, 1971; Deutsch and Suzuki,

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