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SHORT COMMUNICATIONS

Role of *n*-butyl group of buformin in modifying the conformations of poly-L-lysine

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Buformin hydrochloride (BFHCl*), a derivative of GnHCl, is used clinically in the treatment of diabetes and is a powerful denaturing agent of proteins [1]. Conformational changes of proteins are known to determine their interaction with many ligands [2–5]. However the action of BFHCl on the conformation of proteins is difficult to determine as proteins can be folded in five conformations which are present to different extents [6]. To overcome this problem, the effect of BFHCl and GnHCl was studied using CO of PLL, since this polypeptide can be produced such that it exists in solution completely, in α-helix, anti-parallel β-sheets or in a completely random conformation [7–9]. Consequently, the role of BFHCl and GnHCl in disrupting these three different types of conformation could be studied quantitatively and the concentrations required to produce 50% conformational modification determined. It has been shown previously that some biological activities of PLL are related to its conformational configuration [10].

Materials and Methods

Reagents. Redistilled, deionized water was used throughout this work. All the other chemicals used were of analytical grade and were employed without further purification. Buformin hydrochloride was supplied by the Kodama Chemical Co. Ltd, Tokyo, Japan. PPL HBr (M_r 25,000) and GnHCl were obtained from the Sigma Chemical Co., St Louis, U.S.A. The concentration of PLL was 0.033% as determined by a microburet procedure [11]. All solutions were made with water and adjusted to the desired pH with an aliquot of 1.0 M HCl or 1.0 M NaOH. One hundred percent homogeneous α-helix, anti-parallel β-sheet and random conformations of PLL were prepared according to the method of Greenfield and coworkers [8, 9].

CD measurements. CD measurements were made at 25° with a JASCO-600 (Japan) spectropolarimeter calibrated with (+)-D-camphorsulfonic acid ammonium salt and equipped with a computerized data processing system. All spectra were recorded in a square quartz cell with a 1.0 mm path length using band width 1.0 nm, slit width auto, step resolution 0.4 nm and time constant 2.0 sec. Results are expressed as molecular residue ellipticities, $[\theta]$(deg cm^2/dmol), calculated with reference to the PLL concentrations, using a molecular residue of 128. Each CD spectrum reported is the average of four scans. The apparent helical content of each spectrum was estimated as the percent of molecular residue ellipticity at the maximum peak of each spectrum against the residue ellipticity of 100% homogeneous conformation [8, 9].

Results and Discussion

The effect of BFHCl and GnHCl on the completely homogeneous α-helix, β-sheet and random conformations of PLL was followed qualitatively with a CD spectropolarimeter in the wavelength region of 200–250 nm. The CD spectra of PLL in homogeneous α-helix and β-sheet conformations showed negative peaks at elliptical strengths of $[\theta] = -3.8 \times 10^{-4}$ and $[\theta] = 1.9 \times 10^{-4}$ at 221 and 218 nm, respectively, as shown in Figs 1A and 2A. This is in agreement with a previous report by Greenfield and coworkers [8, 9]. PLL spectra in the presence of various concentrations of BFHCl and GnHCl were subtracted from the spectrum obtained in the absence of their reagents to determine the effects of BFHCl and GnHCl on their conformers. The CD curves of both α-helix and β-sheet

* Abbreviations: CD, circular dichroism; PLL, poly-L-lysine; BFHCl, buformine hydrochloride; GnHCl, guanidine hydrochloride.

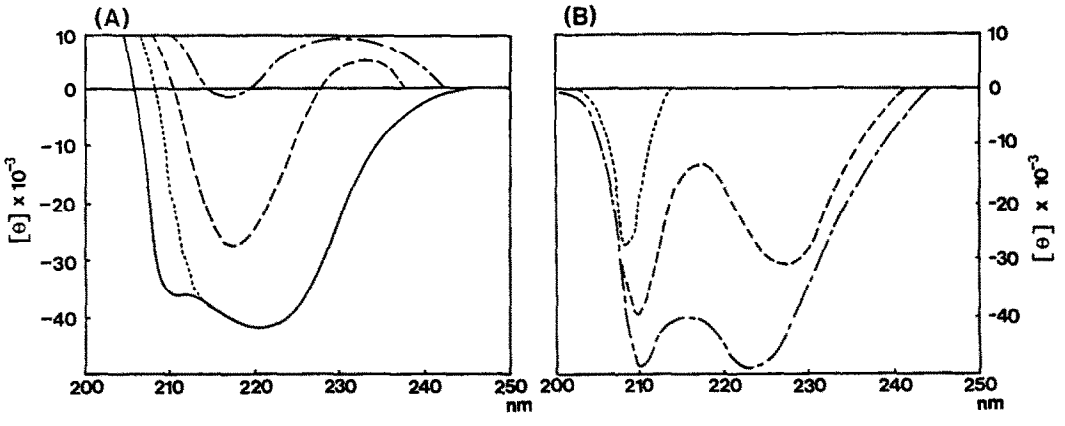

Fig. 1. (A) CD spectra of the α-helix conformer of PLL at pH 11.0 in the presence of various concentrations of BFHCl; and (B) CD spectra subtracted in the presence of various BFHCl concentrations from a spectrum in the absence of its reagent in the 200–250 nm region at 25°. No BFHCl, (——); 1.0 mM, (· · · · ·); 2.0 mM (– – – –); 3.0 mM, (— – —).

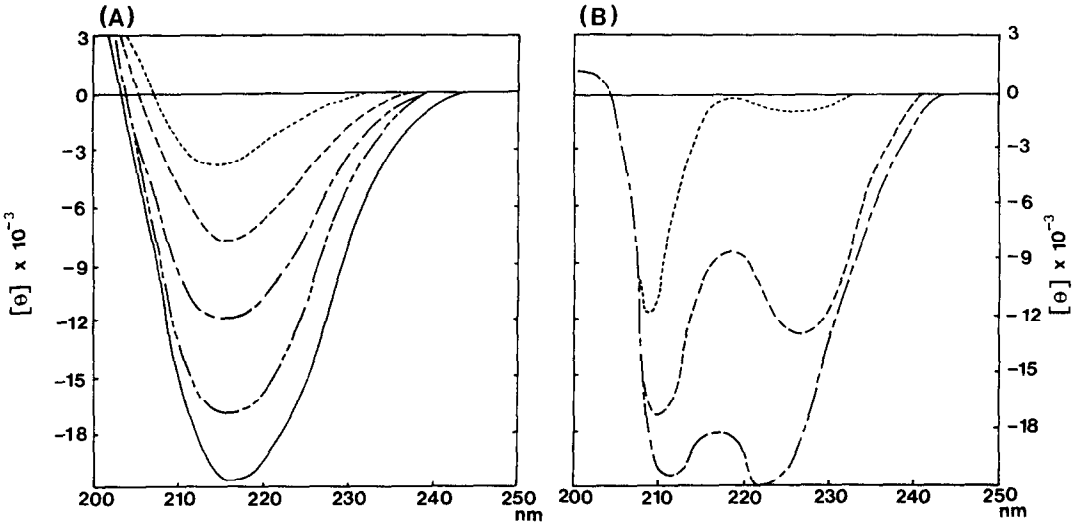

Fig. 2. CD spectra of the β-sheet conformers of PLL subtracted in the presence of various (A) GnHCl and (B) BFHCl concentrations from a spectrum in the absence of their reagents in the 200–250 nm region at 25°. (A) No GnHCl, (——); 0.1 M, (· · · · ·); 0.25 M, (– – – – –); 0.5 M, (— – —); 1.0 M, (— – – —). (B) 1.0 mM, (· · · · ·); 2.0 mM, (– – – – –); 3.0 mM, (— – —).

conformations showed decreases in proportion to the increases in BFHCl (Figs. 1 and 2) and GnHCl (Fig. 2) concentrations. In Fig. 3, the apparent contents of α-helix and β-sheet conformers estimated by the elliptical strength in the presence of various concentrations of GnHCl are shown. When comparing the effect of GnHCl on the α-helix and β-sheet conformations, the concentrations needed for 50% conformational modification were nearly equal at 450 mM. On the other hand, the concentration required for 50% conformational modification by BFHCl was 1.7 mM and the value of BFHCl was 265 times smaller than that of GnHCl (Table 1). The subtracted CD spectra of PLL in the presence of BFHCl (Figs 1B and 2B) showed double maximal peaks because the CD spectra below 210 nm were markedly weakened or disturbed by the reagent, regardless of its much smaller concentration (1/100th that of GnHCl). This demonstrated the difference in effect of BFHCl and GnHCl on both conformations in the shorter wave length region. These results clearly indicate that BFHCl has much stronger effects on α-helix and β-sheet conformers than GnHCl (Table 1).

As shown in Table 1, the 50% modified values of BFHCl were much smaller than those of GnHCl and NaSCN [12] which showed biological activities [13–15]. The active concentration of BFHCl is 265 times lower than that of GnHCl. Another remarkable difference in the behaviour of BFHCl was that the initial concentration needed for modification of the random conformation was 1.0 mM, whereas GnHCl had absolutely no effect up to 6.0 M on this conformation [12]. Thus, the most disordered form of PLL, which is naturally postulated to be the most stable form, was modified by BFHCl as effectively as were the α-helix and β-sheet conformations. Modification of the random conformation of PLL has been reported with sodium dodecyl sulfate at low concentrations [16, 17], but with other salts molar concentrations were needed [18]. The lower concentration required for conformational change by BFHCl in comparison with these salts and GnHCl may be due to hydrophillic and hydrophobic micelles formed by BFHCl.

Considering the stabilizing factors of each structure of PLL [19], α-helical PLL is stabilized largely by intramolecular hydrogen bonds while the β-sheet conformer

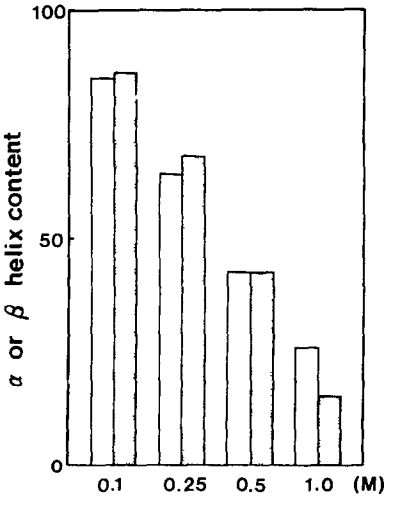

Fig. 3. α-Helix (open) and β-sheet (closed) conformation contents of PLL calculated from the CD elliptical strength of a maximal peak in the presence of various concentrations of GnHCl.

owes a large part of its stability to hydrophobic interactions between lysyl residues by intermolecular aggregation. The almost equal concentrations needed for 50% modification by BFHCl of α-helix and β-sheet conformations suggest that the effect of BFHCl is evenly attributable to disruptions of hydrophobic interaction, intermolecular aggregation and/or the hydrogen bonds contributing to the stability of the conformations, the integrity of which depends mainly upon maintenance of C=O···NH bonds. That is to say, the effect must be due to the amphoteric properties of BFHCl with *n*-butyl group and guanidine in a molecule. It is worth noting that this equal effect of BFHCl on α-helix and β-sheet conformations might be due to hydrophillic and hydrophobic micelle formation, and therefore differ

Table 1.

Salt used	Concentration to disrupt (mM)		Reference
	α Conformer	β Conformer	
NaCl	5600	6500	18
KCl	8000		12
CsCl	1600	3000	18
LiCl	3800	3100	18
NaSCN	11	9	12
Urea	800	3000	12
GnHCl	450	450	
Buformin	1.7	1.7	

The concentrations required for 50% conformational modification of α-helix and β-sheet conformers of PLL as derived from CD.

from that of other salts with only hydrophilic ions which showed different effects on the respective conformations at different concentrations (Table 1). A compound with amphoteric properties at an adjacent position in a biguanide derivative might easily provide two kinds of micelle, spherical and reversed, due to spontaneous association leading to modification of α-helix and β-sheet conformations stabilized by inter- and intramolecular hydrogen bonds. These findings on the strong denaturation effect of BFHCl with *n*-butyl group on the α-helix, antiparallel β-sheet and random conformations may prove helpful in the development of more effective anti-diabetic drugs.

In summary, this investigation using CD determined the effects of BFHCl and GnHCl in disrupting the conformation of PLL. BFHCl was active at 1/265 of the concentration of GnHCl in disrupting both the α-helix and β-sheet conformation of PLL. BFHCl also disrupted the random conformation of PLL which was never seen on treatment with GnHCl. The very potent activity of BFHCl in disrupting the conformation of the polypeptides may be caused by its capacity to form amphoteric micelles. The anti-diabetic activity of BFHCl may also be related to micelle formation and/or protein denaturation.

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Biochemical and biological properties of methotrexate analogs containing D-glutamic acid or D-erythro,threo-4-fluoroglutamic acid

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Structural modification of existing antifolates may create new agents with altered therapeutic effects [1]. For example, substitution of an amino acid analog for L-glutamate (Glu) in classical antifolates may alter enzyme inhibition, transport properties, or the ability to form poly-γ-glutamate metabolites [1]. Often, the amino acid analog chosen is only or most readily available as the D,L-racemate. Thus, the D,L-racemate may be used to synthesize the analog first; if interesting biological results are obtained, the analog containing the L-enantiomer may be prepared. It is generally assumed in studies using a D,L-racemate that the D-enantiomer-containing analog is inactive and does not interfere with effects of the L-enantiomer-containing species. In the case of methotrexate (MTX*), this assumption has been validated only for D-MTX compared to L-MTX [2].

We previously studied D,L-*e,t*-γ-fluoroMTX (4-amino-10-methylpteroyl-D,L-*erythro,threo*-4-fluoroGlu; D,L-*e,t*-FMTX), an MTX analog in which L-Glu is replaced by D,L-*erythro,threo*-4-fluoroGlu, and its constituent diastereomers D,L-*e*-FMTX and D,L-*t*-FMTX [3–5]. Based on published studies of D-MTX [2], we assumed that the D-enantiomer-containing species were essentially inactive. However, we remained concerned about the remote possibility that fluorine substitution might alter enantiomeric specificity in our test systems. To address this concern, we enzymatically prepared D-*e,t*-FMTX and studied its activity. We included D-MTX in these studies to expand the data base on this contaminant found in clinical MTX preparations [2].

Materials and Methods

L-MTX was a gift of Lederle (Pearl River, NY). D,L- and D-MTX were from Aldrich Chemicals (Milwaukee, WI). 4-Amino-10-methylpteroyl[γ-(1*H*-tetrazolyl- 5-yl)-L-α-amino butyric acid] [6] was a gift of Dr. T. Kalman (SUNY, Buffalo, NY). 4-Amino-10-methylpteroyl-D,L-(3-hydroxy-Glu) and 4-amino-10-methylpteroyl-D,L-(4-methylene-Glu) [7] were gifts of Dr. M. G. Nair (University of South Alabama, Mobile). Other chemicals were reagent grade or higher.

D-*e,t*-FMTX was prepared by exhaustive digestion of D,L-*e,t*-FMTX [3] with carboxypeptidase G_2 (CPG_2), which specifically releases L-amino acids from pteroates (*vide infra*). D,L-*e,t*-FMTX (20 μmol) was hydrolyzed (37°) by 40 I.U. of CPG_2 in 25 mM Tris–Cl, pH 7.3 and 0.1 mM $ZnCl_2$ (200 mL). After no further absorbance change at 320 nm was observed (t = 15 min), incubation was continued for 30 min. Based on ΔA_{320} and the $E_{320,\,pH\,7.3}$ for production of 4-amino-10-methylpteroate [8], 49% of the substrate was hydrolyzed. The resulting solution was chromatographed in two portions on DE-52 (0.7 × 21 cm; Whatman, Clifton, NJ) equilibrated at 4° with 50 mM NH_4HCO_3, pH 8.0. After loading and washing with 70 mL of initial buffer, each column was eluted with a linear gradient (500 mL total) from 50 to 200 mM NH_4HCO_3, pH 8.0. 4-Amino-10-methylpteroate, identified by its UV spectrum at pH 13 and HPLC retention time [9], was well resolved from D-*e,t*-FMTX. Fractions containing material with a UV spectrum and HPLC retention time similar to D,L-*e,t*-FMTX were lyophilized. Exhaustive CPG_2 digestion of this material showed it contained <4% of the L-isomer (D-*e,t*-FMTX does not inhibit CPG_2; *vide infra*).

Radiochemicals. L-[3′,5′,7′,-^{3}H]MTX (20 Ci/mmol) and [5-^{3}H]dUrd (22 Ci/mmol) were from Moravek Biochemicals (Brea, CA). The purity of L-[^{3}H]MTX was assessed by HPLC [4].

Enzymes and assays. CPG_2 was purified [10] from *Escherichia coli* harboring a plasmid containing the *Pseudomonas* CPG_2 cDNA [11] and assayed as described [8], except that 100 μM L-MTX was used. Dihydrofolate reductase (DHFR; EC 1.5.1.3) was partially purified from CCRF-CEM cells and assayed as described [6]. Drug concentrations inhibiting DHFR activity (1.6×10^{-3} I.U.) by 50% (IC_{50}) were determined as described [6]. L-[^{3}H]-MTX uptake by CCRF-CEM cells was measured as described [4].

Cell culture. Human T-lymphoblastic CCRF-CEM [12] and sublines MTX resistant via decreased transport [13] or DHFR increase [14] were cultured in RPMI 1640 containing 10% horse serum (GIBCO) and additions as indicated [4]. Cell outgrowth inhibition and drug concentration inhibiting cell growth by 50% (EC_{50}) were determined as described [6]. CCRF-CEM cells used as a DHFR source and to determine EC_{50} were *Mycoplasma* free (Gen-Probe Inc., San Diego, CA). Studies on thymidylate (dTMP) biosynthesis and inhibition of [^{3}H]MTX uptake were completed within 11 days and 2 months, respectively, of this negative test; testing 10 months later showed contamination in all lines. D-*e,t*-FMTX was depleted prior to this discovery so the studies could not be repeated. However, since cells grew normally during the studies

* Abbreviations: MTX, methotrexate; D,L-*e,t*-γ-fluoro-MTX (D,L-*e,t*-FMTX), 4-amino-10-methylpteroyl-D,L-*erythro,threo*-4-fluoroGlu; CPG_2, carboxypeptidase G_2; DHFR, dihydrofolate reductase; and dTMP, thymidylate.