On how a myosin tryptophan may be perturbed

DONALD B. BIVIN*, SHIGEO KUBOTA†, ROBERT PEARLSTEIN‡, AND MANUEL F. MORALES*

*Department of Physiology, University of the Pacific, 2155 Webster Street, San Francisco, CA 94115; †The Peninsula Company, Belmont, CA 94002; and ‡Division of Computer Research and Technology, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT A well-known indication that a nucleotide has bound to myosin is the enhancement of the fluorescence of a specific tryptophan in the "subfragment 1" segment of the protein. Empirically the effect has been enormously useful in myosin enzymology. But beyond an early suggestion that it arises from a purine-tryptophan charge-transfer complex, the mechanism of the effect has not been considered. Here we consider the alternative that it arises from an ionizable group (either another residue or the phosphate of the nucleotide) whose proximity to the tryptophan is altered by substrate binding. We study this possibility by studying the interaction of an ionizable residue and tryptophan when both are incorporated in a diketopiperazine structure. The geometry of the situation is inferred from molecular mechanics simulations. Unexpectedly, the best explanation seems to be that the field of the imposed charge, acting across space, affects events in the excited state of the indole.

The perturbation of the absorbance (1) and emission (2) of a tryptophan residue by the binding of the nucleotide substrate to the ATPase site of myosin "subfragment 1" has been empirically useful in analyzing the kinetics of the enzyme (3), but it was not until recently that attempts to identify the residue (refs. 4 and 5 suggest that it is Trp-510) or to relate it structurally to the bound substrate (suggestions about this relation are in refs. 6 and 7) have been made. It is possible that the negatively charged polyphosphate tail of bound ATP or ADP is near Trp-510. However, the physical reason for the considerable fluorescence enhancement accompanying ATP binding or the lesser one upon ADP binding has not been established. An early suggestion was that the tryptophan indole ring forms a charge-transfer complex with the purine ring of ATP (8), but alternative explanations have not been considered. We undertook the present study, hoping to get clues about the aforementioned effect in myosin and, more generally, information about environmental effects on tryptophan. Our specific goal was to study the effect of a single charge positioned in a known relation to the indole ring, using a setup in which the sign and quantity of the charge (and to a limited extent the geometry of the arrangement) could be varied. We sought to accomplish this goal by using diketopiperazines constructed from tryptophan and the L or D forms of amino acids such as histidine or aspartic acid, using appropriate molecular mechanical analysis to characterize the charge-indole relation.

MATERIALS AND METHODS

Preparation of the Diketopiperazines. The strategy followed was to prepare *t*-butoxycarbonyl (Boc)-protected amino acids, to couple these into linear dipeptide esters, and then to cyclize them and remove the protective groups.

Boc-Trp and Boc-Asp(β -OBz) (where Bz = benzoyl) were prepared according to Morodes *et al.* (9). Glu(γ -OBz) OBz-

TosOH and His-OMe-2HCl were prepared according to Zervas *et al.* (10) and Humphlett and Wilson (11), respectively. Linear protected dipeptide esters were prepared by the *N,N'*-dicyclohexylcarbodiimide/*N*-hydroxysuccinimide method (12). Dimethylformamide was the solvent for coupling. The products were purified from ethyl acetate petroleum ether, except Boc-L-Trp-L-His-OMe, which was purified from warm ethanol. In TLC, each of the products gave a single spot (ninhydrin negative) using the KI/toluidine reaction for detection (13). To prepare the diketopiperazines, a Boc group was removed by dissolving 10 mmol of Boc-protected dipeptide ester in 120 ml (240 ml for the histidine-containing dipeptide) of 0.1 M HCl/96% formic acid (14) and incubating for 30 min at room temperature. The resulting clear solution was condensed to dryness *in vacuo* (<30°C), leaving the theoretical yield of Boc-free dipeptide ester. This residue was dissolved in 150 ml of 0.1 M acetic acid/2-butanol containing 1.1 ml or 10 mmol (2.2 ml for the histidine-containing dipeptide) of *N*-methylmorpholine, and the solution was refluxed for 3 hr (15). The solution was condensed to $\approx$ 50 ml and cooled overnight at 4°C. The precipitates were filtered, washed with water, and dried *in vacuo*. The crude cyclic peptides were purified from isopropanol, except the histidine-containing ones, which were purified from warm water. The Bz groups on the side chains of aspartic acid and glutamic acid were removed by catalytic transfer hydrogenation (16) as follows. To 2 mmol of cyclic dipeptide in about 50 ml of dimethylformamide were added $\approx$ 300 mg of 10% Pd on charcoal and 400 mg of ammonium formate. After stirring for 3–5 hr, precipitates were filtered and washed with $\approx$ 50 ml of water. Combined filtrates were condensed to dryness *in vacuo*, leaving a white powder of Bz-free cyclic peptide. A solution of crude cyclo(Asp-Trp-) in $\approx$ 50 ml of water was adjusted to pH 3.0 with dilute aqueous citric acid, lyophilized, and purified once more from water/ethanol. Crude powder of cyclo(Trp-Glu-) was suspended in $\approx$ 50 ml water, and the system was adjusted to pH 3.0 with dilute aqueous citric acid. After an overnight stand at 4°C, precipitates were collected and purified from $\approx$ 100 ml of warm water. In TLC cyclo(Asp-Trp-) and cyclo(Trp-Glu-) each gave a single, strongly fluorescing spot (ninhydrin negative) when tested with the KI/toluidine reaction (13). Some characteristics of the compounds are in Table 1.

For the foregoing syntheses, the following precursors were obtained from Aldrich: L-tryptophan, L- and D-aspartic acid, L- and D-glutamic acid, and L- and D-histidine. Diphenyl chlorophosphate, di-*tert*-butyl dicarbonate, *N,N'*-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, 96% formic acid, *N*-methylmorpholine, 2-butanol, 10% Pd on activated charcoal, and ammonium formate were obtained from Aldrich. Sigma supplied L-Trp-OMe-HCl and *N*-acetyl-L-tryptophan ethyl ester.

Preparation of Samples for Spectroscopic Measurements. Samples were dissolved either in doubly distilled water or in 5 mM sodium citrate/10 mM potassium phosphate buffer. In some cases, the samples were heated at 50°C for 1 hr to

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Abbreviations: Boc, *t*-butoxycarbonyl; Bz, benzoyl.

Table 1. Properties of diketopiperazines used in this work

Cyclo compound	R_f^*	$[\alpha]_D^{25}$	Solvent [†]	Yield, %
L-Trp-L-His	0.57	-94.1	1% MeOH	77
L-Trp-D-His	0.57	+20.9	1% MeOH	72
L-Trp-L-Glu	0.80	-27.5	0.5% H ₂ O (pH 7)	96
L-Trp-D-Glu	0.80	+47.6	0.5% H ₂ O (pH 7)	93
L-Trp-L-Asp	0.80	-36.9	0.5% H ₂ O (pH 7)	93
D-Trp-D-Asp	0.80	+28.1	0.5% H ₂ O (pH 7)	91

$[\alpha]_D^{25}$, specific optical rotation.

* R_f was measured on Merck silica gel 60 F-254, using a solvent system of isopropanol/acetic acid/water (3:1:1, vol/vol).

[†]Solvent used for the $[\alpha]_D^{25}$ measurement.

facilitate solvation. Sample concentration, gravimetrically determined, was 1 mM, 100 μ M, or 50 μ M. To minimize the manipulations of any given sample and to minimize dilution, aliquots were taken from a common bath of dissolved material, and the pH of each was set separately. The absorbance and fluorescence spectra of each aliquot were then taken. Absorbance spectra were acquired on a Cary 118C spectrophotometer. Fluorescence spectra were acquired on an SLM 8000 spectrofluorometer. Samples were excited at 280 nm with a band width of 8 nm, and the emission spectrum was acquired with a band width of 1 nm at 1 sec/nm. Four spectra were averaged to produce the final emission spectra. To produce the fluorometric titration curves, samples were excited at 280 nm with a band width of 8 nm, and their emission was monitored at 350 nm with a band width of 8 nm. Data were acquired at 10 sec per point and averaged for 5 min; 4 $\times$ 10 mm cuvettes were used throughout, with excitation along the 4-mm direction. In all cases, the polarization of the exciting beam was at 55° to the vertical, and emission was monitored at 0° with respect to vertical. All spectra were taken at 20°C. The raw data were transferred to an IBM PC-type computer and plotted using the plotting routines of GRAPHIER from Golden Software. Background subtraction was performed, and the resulting net fluorescence amplitudes were then plotted against pH, again using the GRAPHIER routines.

CD Measurements. In an effort to test for intermolecular interactions between the diketopiperazine molecules in solution, we searched for evidence of exciton splitting in the spectra of the compounds recorded at 1 mM and 0.1 mM. These measurements were conducted in a modified Cary 60 spectropolarimeter graciously made available to us by Marcos Maestre (Lawrence Berkeley Laboratory).

Molecular Mechanics. For the most part, we were concerned with measuring intramolecular distances (e.g., from the centroid of the ionizable residue on the amino acid mate to the NH or to the C-4 of the tryptophan indole) in the "most likely" conformations of the particular diketopiperazine under study. Most likely conformations were judged to be conformations of low energy. The set of these consisted of the conformation found that had the lowest energy, as well as conformations with energies differing from the lowest by 20 kcal per mol or less. The search for such conformations was performed with the program SYBYL 6.0 (Tripos Associates, St. Louis). For each conformation, we tabulated the vector distances from the centroid of the ionizable group to various positions on the indole ring. The entire procedure was carried out for the charged and uncharged states of the ionizable group and for the L and D enantiomers of the amino acid mated with L-tryptophan. The procedure was initiated by using a model in which Cl atoms were substituted for the residues at the two α -carbon atoms (generating steric bulk without the complication of additional degrees of freedom), and electrostatic effects were ignored. After breaking one of the ring bonds, full-circle rotation (valence angle constant) was allowed to occur in 10° steps; after each step, it was

ascertained whether the ring closed, using the systematic search option of SYBYL. In the case of cyclo-(Trp-Asp-), for example, 18 closed conformers were found for the L-aspartic acid compound, and 15 were found for the D-aspartic acid compound. Each of these discrete structures became a starting conformation for energy minimization, using the Maximin2 option (Broyden-Fletcher-Goldfarb-Shanno minimizer, gradient termination). All starting conformations minimized to two unique, boat-like ring conformations characterized by opposite directions of ring puckering (Cl substituents pseudoaxial as "beta-positive" or pseudoequatorial as "beta-negative" or in combination in the L/L or D enantiomer). Conformational energy space was searched for each of the two isomers with the actual residues in place. The systematic search option was used to generate starting conformations resulting from rotations of both α -carbon bonds in 30° steps about a full circle. In this case, an electrostatic energy term was included. Partial atomic charges were assigned using the Gasteiger-Marsili method, assuming a dielectric constant proportional to distance between the interacting atoms. The conformations chosen in this way were then used to initiate energy minimization using the Maximin2 option. The minimal energy conformations, using beta-positive and beta-negative ring conformers, were superimposed at their common indole nucleus, and the various distances were calculated.

We also made a few simulations of the situation in which a negative point charge (centroid of a carboxylate group) was in the plane of the indole, near C-7, and computed the electrostatic potential along the transition dipole (17, 18). These simulations showed several changes in sign of the potential, and they also suggested that the conformations in which the carboxylate centroid assumes this location are more numerous in cyclo-(L-Trp-L-Glu-) than in cyclo-(L-Trp-D-Glu-).

RESULTS AND DISCUSSION

Our experimental arrangement enabled us to control the ionization state of a group near the indole moiety of tryptophan, and, because we dealt with L and D enantiomers of the amino acid paired with tryptophan, it enabled us to compare two geometrically different relative locations of the group. We found early on that varying the ionization state has no detectable effect on the *absorption* spectrum of the diketopiperazines, so we must conclude that the effects to be reported relate solely to the emission process.

Next we found (as is indicated in the literature) that protonation appeared to blueshift the *emission* spectrum by

FIG. 1. Fluorescence emission spectra of cyclo-(L-Trp-D-His-) at pH 8.10 (curve A) and pH 4.26 (curve B).

FIG. 2. Fluorescence emission intensity as a function of pH for a "control" compound (*N*-acetyl-L-tryptophan ethyl ester; *) and for diketopiperazines in each of which L-tryptophan is paired with one of the following amino acids: ○, D-histidine; ●, L-histidine; ■, D-aspartic acid; □, L-aspartic acid; ☆, D-glutamic acid; ★, L-glutamic acid.

a few nanometers (Fig. 1)—a fact that could be expressed as a relative change in the contributions of two superimposed distributions. In all subsequent experiments, we were primarily concerned with the emission intensity at 355 nm, specifically, with how this parameter varies with pH.

To compare one curve with another using data taken on different occasions, we took pains to establish the high pH asymptote of each curve and considered the ratio between two such asymptotes to be the normalization factor between their respective titration curves. The raw data for the six curves are shown in Fig. 2. This data shows that with increasing hydrogen ion concentrations corresponding to pH values below ≈ 3 intensities decrease (reversibly) more rapidly than expected from the titration of a single group. This suggests that the increasing free proton concentration is exerting a quenching effect, by perturbing the indole in the excited state either by binding of the proton to the excited indole or by abstracting an electron from the excited indole (19–21). To circumvent this complication, we titrated a control tryptophan-containing compound with no dissociable groups—namely, *N*-acetyl-L-tryptophan ethyl ester—and observed a qualitatively similar quenching with increasing H^+ concentration. This additional data allowed us to "correct" the diketopiperazine data. Since different indole-containing compounds have different absolute emission intensities, we

FIG. 3. The fluorimetric diketopiperazine titration curves depicted in Fig. 2 have been corrected for free proton effect (see text), then fitted by a least-squares procedure. The theoretical dissociation curve for each compound (same symbols as in Fig. 2) is then plotted, along with the corrected experimental data, as a continuous line. Corresponding to these dissociation curves are the pK values for the diketopiperazine in which L-tryptophan is paired with D-histidine (6.55), L-histidine (6.45), D-aspartic acid (4.20), L-aspartic acid (4.25), D-glutamic acid (4.80), and L-glutamic acid (4.60).

corrected the diketopiperazine curves for the same percent quenching observed in the control compound. The corrected titration curves now assumed the character of single ionization curves. Accepting the experimentally established high pH asymptote and the premise of a simple acid dissociation curve, we found in each case the best-fitting (least-squares) curve to represent the data. Fig. 3 shows that all the data are thus well represented. The pK values so deduced are in the figure legend. It is obvious from their magnitudes that they refer to the amino acids paired with tryptophan. A primitive analysis of our results is in Table 2. The raw effect of acid ionization on emission is measured by the amplitude of the titration curve. However, since compounds in corresponding states differ markedly from one another in their emissions, we must divide each amplitude by, say, the charged level to obtain a comparable "effect." Column D in Table 2 shows that the effect of ionization is very strong. Furthermore, we can crudely deduce a "geometric" effect as a difference in ionization effect in the L and D enantiomers. The only significant difference seems to be that between the D-glutamate-containing compound and all the others. Another early concern in our work was that our diketopiperazines might engage in an ionization-dependent Trp-Trp dimerization. To

Table 2. Empirical implications of the measurements

Cyclo compound	A	B	C	D	E
L-Trp-D-His	114	438	-324	-74	
L-Trp-L-His	173	704	-531	-75	0.014
L-Trp-L-Asp	560	330	230	70	
L-Trp-D-Asp	612	285	327	71.5	-0.45
L-Trp-L-Glu	518	313	205	65	
L-Trp-D-Glu	312	230	82	26	0.30

Each titration curve in Fig. 2 specifies the asymptotic emission intensity level (in arbitrary units) when the diketopiperazine bears no net charge (A) and when it bears a full unitary charge (B), be it positive or negative. The quantity, $C = A - B$, is thus the amplitude of the curve. Since the compounds differ in their uncharged levels (A), the quantity, $D = 100(C/A)$, expresses the maximum percent effect of having a charge near the indole ring. To a first approximation, a comparison of the levels of two charged (L vs. D) isomers, each normalized by its respective uncharged level, expresses "geometrical" factors in the charge effect; column E gives the differences (in the order L minus D) in B/A for each of the three isomer pairs.

examine this contingency, we studied the CD spectra of our compounds at concentrations of 0.1 mM and 1 mM, looking for a negative band in the 280–290 nm L_B region (evidence of exciton splitting). No such band appeared in the spectrum of cyclo(-Trp-Asp-) or cyclo(-Trp-Glu-); there is such a band at 289 nm in cyclo(-Trp-His-), but it is known to arise from a dipole-dipole interaction between the indole and imidazole rings and is too small to indicate exciton splitting. Similar tests leading to the same conclusion—no Trp-Trp interaction—were kindly conducted on our behalf by Curtis Johnson (Oregon State University). Accordingly, we decided that we could consider exclusively intramolecular explanations.

The traditional, and quite logical, way to interpret curves such as those of Fig. 3 is to think that the protonated species of each mated residue is capable of quenching the fluorescence of the tryptophan. After taking this view comes the question of what mechanism mediates the quenching. One plausible mechanism is the formation of a hydrogen bond with, say, the pyrrole nitrogen atom of the tryptophan indole. Support for this idea is that emission is reduced when the water solvent is replaced by isobutanol, even though the replacement might have been expected to suppress dielectric relaxation. Another widely considered and plausible mechanism is quenching by the transport of charge—protons through space and electrons through bonds or through space (19, 20, 22). For example, in tryptophan itself a proton from the α -amino group might be donated to the C-4 of the indole [the work of Barkley and her associates (23, 24) has forced a change in this simplest hypothesis; it must now be surmised that a protonated carboxyl or amino group closely approaches the C-4 locus (25), enabling a proton or an electron to move through space to effect the quenching].

Fully expecting to explain our phenomena by either of the foregoing hypotheses, we were surprised by contradictions that our other results seem to pose. If proton donation was involved in the quenching mechanism, one might expect that substitution of deuterons for protons would evoke a significant effect. However, when we studied, for cyclo(-L-Trp-L-His-) and cyclo(-L-Trp-D-Asp-), the ratio of intensities in $^2\text{H}_2\text{O}$ and H_2O at high and low pH asymptotes, we obtained 1.17 and 1.16, and 1.21 and 1.20, respectively; in other words, we found *no* isotope effect. Still more disturbing are the results from molecular mechanics. In all of our diketopiperazines the "through bonds" path between the ionizable group

FIG. 4. Distances between the carboxylate and indole centroids in minimal energy conformations of cyclo(-L-Trp-L-Asp-). The indole ring is viewed edge-on. The conformation of lowest energy (found by the procedure described in the text) is depicted in "stick-model" fashion, and the vector colored purple connects its two centroids. The other, minimal energy, conformers are not shown; only their corresponding vectors are shown. Taking the energy of the lowest energy conformer as zero, the conformer with the blue vector has an energy of 0–0.5 kcal/mol, that with the cyan vector has 0.5–1.0 kcal/mol, that with the blue-green vector has 1.0–2.9 kcal/mol, that with the white vector has 2.0–5.0 kcal/mol, and that with the orange vector has 5.0–10.0 kcal/mol.

and indole is nonresonating and may be implausibly long for the conduction of electrons. In addition, in the sets of minimal energy conformations, the distances between the ionizable group and key loci on the indole also seem much too long, even for hydrogen bond formation. For example, in Fig. 4, which refers to cyclo(-L-Trp-L-Asp-), the *shortest* vector joining the carboxylate centroid to the indole centroid is 5.7 Å. The same circumstance is expressed by the data from cyclo(-L-Trp-L-Glu-). If the 100 conformations differing from the conformation of minimum energy by 5 kcal/mol or less are treated as effectively isoenergetic, and if then there is constructed the distribution of distances from the carboxylate centroid to C-4, then the modal distance is 9.5 Å and the shortest distance is 3.5 Å.

The interpretational dilemma might be resolved in one of two ways. In one we could declare that for the resolution we do not possess the required data (for example, say that in the excited state forces generated in the indole attract the ionizable group, thus achieving atomic contact, or perhaps say that the transition responsible for the quenching is so fast that it always occurs even though atomic contact is a very rare event). In the other way, we could embrace an unorthodox idea: we could suppose that the electric field from a vicinal positive charge decreases fluorescence intensity and that the electric field from a vicinal negative charge increases it. These opposite effects might arise naturally from directional effects going on in the indole ring (and it was for this reason that we cursorily studied the field of the charge at the indole ring).

Although we did not become aware of their paper until this work was virtually finished, and although we reached an opposite conclusion, we note that the priority for exploiting the convenient properties of diketopiperazines in this manner belongs to Shinitzky and Goldman (26). In regard to the perturbation of a particular tryptophan residue of myosin subfragment 1 by the binding of a nucleotide, we note that the results found here make it clear that the proposal of an adenine-tryptophan charge-transfer complex by Morita and coworkers (8), while possible, is not unique. For example, the field of negative charge around bound ATP or ADP might effect the enhancement directly, or the conformational change caused by the binding might indirectly influence the immediate environment of the particular tryptophan.

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1. Morita, F. (1967) *J. Biol. Chem.* **242**, 4501-4506.
2. Werber, M. M., Szent-Gyorgyi, A. G. & Fasman, G. D. (1972) *Biochemistry* **21**, 1284-1294.
3. Trentham, D. R., Eccleston, J. F. & Bagshaw, C. R. (1976) *Q. Rev. Biophys.* **9**, 217-281.
4. Johnson, W. C., Bivin, D. B., Ue, K. & Morales, M. F. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9748-9750.
5. Hiratsuka, T. (1992) *J. Biol. Chem.* **267**, 14949-14954.
6. Botts, J., Thomason, J. F. & Morales, M. F. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 2204-2208.
7. Morales, M. F., Ue, K. & Bivin, D. B. (1992) in *Sliding Filament Mechanisms*, ed. Sugi, H. (Plenum, New York).
8. Yoshino, H., Morita, F. & Yagi, K. (1972) *J. Biochem.* **72**, 1227-1235.
9. Morodes, A., Hallett, A., Wunsch, E., Keller, O. & Wersin, G. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 1651-1653.
10. Zervas, L., Winitz, M. & Greenstein, J. P. (1961) *J. Org. Chem.* **22**, 1515-1521.
11. Humphlett, W. J. & Wilson, C. V. (1961) *J. Org. Chem.* **26**, 2507-2510.
12. Weygand, F., Hoffman, D. & Wunsch, E. (1966) *Z. Naturforsch. Teil B* **21**, 426-428.
13. Arx, E. & Neher, R. (1963) *J. Chromatogr.* **12**, 329-341.
14. Terada, S., Kato, K., Kato, N. & Izumiya, N. (1981) *J. Pept. Protein Res.* **15**, 441-454.
15. Suzuki, K., Sasaki, Y., Endo, N. & Mihara, Y. (1981) *Jpn. Pharmacol. Bull.* **233**, 233-237.
16. Amwer, M. K. & Spatula, A. F. (1980) *Synthesis* **1980**, 929-932.
17. Yeagers, E. (1968) *Biophys. J.* **8**, 1505-1510.
18. Yamamoto, Y. & Tanaka, J. (1972) *Bull. Chem. Soc. Jpn.* **45**, 1362-1366.
19. Steiner, R. F. & Kirby, E. P. (1966) *J. Am. Chem. Soc.* **73**, 4130-4135.
20. Stryer, L. (1966) *J. Am. Chem. Soc.* **88**, 5708-5712.
21. Vander Donckt, E. (1969) *Bull. Soc. Chim. Belg.* **28**, 69-75.
22. Shizuka, H., Serizawa, M., Shimo, T., Saito, I. & Matsuura, T. (1988) *J. Am. Chem. Soc.* **110**, 1930-1934.
23. Tilstra, L., Sattler, M. C., Cherry, W. R. & Barkley, M. D. (1990) *J. Am. Chem. Soc.* **112**, 9176-9182.
24. Colucci, W. J., Tilstra, L., Sattler, M. C., Fronczek, F. R. & Barkley, M. D. (1990) *J. Am. Chem. Soc.* **112**, 9182-9190.
25. Ricci, R. W. & Nesta, J. M. (1976) *J. Phys. Chem.* **80**, 974-980.
26. Shinitzky, M. & Goldman, R. (1967) *Eur. J. Biochem.* **3**, 139-144.