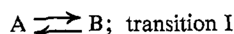
Variability of State A for Some Members of the Chymotrypsinogen Family of Proteins[1]

Sir:

We wish to report some results obtained with the force theory of Brandts[2] which confirm the mathematical form of the theory as a representation of the conformational stability of globular proteins and which lead to some interesting conclusions about the variation in the "native" state (state A) among members of the chymotrypsinogen (CGN) family.

The thermally induced conformational isomerizations of α-chymotrypsin (CT) and dimethionine sulfoxide chymotrypsin (DMSCT) were measured, using ultraviolet difference spectroscopy, at several pH values in the acid pH region. Despite speculations to the contrary,[3] existing evidence strongly supports the conclusion that this transition (transition I) is a first-order cooperative process,[4] *i.e.*, a two-state process is traditionally defined, and that the reaction can be represented as an equilibrium between two thermodynamic states A and B.



$$A \rightleftharpoons B; \text{ transition I}$$

The standard free energy of transition I as a function of temperature and after empirical adjustment to reduce the data to a given pH and ionic strength is very well expressed by Brandts' polynomial[2] written for present purposes as

$$\Delta\bar{F}^\circ = p(N\Delta\bar{h}^\circ - NT\Delta\bar{s}^\circ + A'T + B'T^2 + C'T^3)$$

The coefficients A', B', and C' are related to the hydrophobic contribution to the conformational free energy and are calculated from data for the transfer of nonpolar amino acid side chains from ethanol to water and the amino acid composition of the proteins.[2] A', B', and C' are virtually identical for all members of the CGN family since the amino acid composition is essentially identical. N is the total number of residues in the protein. Least-squares fitting of the experimental free-energy data yields p, $\Delta\bar{h}^\circ$, and $\Delta\bar{s}^\circ$. For the present these may be considered only as fitting parameters, but if the form of the Brandts force theory is correct, all members of a given family should yield the same values for $\Delta\bar{h}^\circ$ and $\Delta\bar{s}^\circ$ for transition I no matter what change in the protein occurs; variation in the thermodynamic behavior of transition I among the proteins should appear primarily in p, the measure of the size of the cooperative unit for the transition.

The results of the analysis of transition I for CGN,[5] CT, and DMSCT are given in Table I. As predicted, $\Delta\bar{h}^\circ$ and $\Delta\bar{s}^\circ$ are essentially constant for all proteins and only the size of the cooperative unit as measured by p is different. It appears the A', B', and C' are uncertain themselves by an unknown temperature-independent factor and hence p has no absolute significance as a measure of the cooperative unit.[6] We can guess that p is approximately twice as large as it should be. Hence the cooperative unit (in terms of fraction of residues) for CT is about 0.5 and for DMSCT and CGN approximately 0.25. From these results we first conclude that the average residue approximation used by Brandts in development of eq. 1 is correct and that the force theory is correct in form for these proteins. As a consequence, the quadratic and cubic terms apply almost entirely to the hydrophobic bonding contribution to the standard free energy and p is correctly a measure of the relative size of the cooperative unit for transition I of each protein.

Table I. Thermodynamic Parameters for Transition I

Protein specie	p	$\Delta\bar{h}^\circ$	$\Delta\bar{s}^\circ$
Chymotrypsinogen[a]	0.63	800	5.1
α-Chymotrypsin	1.01 ± 0.02	780 ± 10	5.1 ± 0.2[b]
Dimethione sulfoxide chymotrypsin	0.59 ± 0.04	790 ± 20	5.2 ± 0.4[b]

[a] From ref. 4. [b] $\Delta\bar{s}^\circ$ corrected for pH dependence to correspond with value given for chymotrypsinogen. "Best fit" parameters; consult ref. 6 for details.

Fluorescence, optical rotatory dispersion, and ultraviolet absorption patterns and sedimentation coefficients indicate that state B is identical for the three proteins.[6] Optical rotatory dispersion and deuterium exchange studies demonstrate that state B, however, retains a significant amount of structural integrity. Although the present evidence is not unequivocal, it is reasonable to conclude at least tentatively that state B is identical for all three proteins. This being the case, the variation in p must be the result of variations in state A and, indeed, these proteins show on comparison differences in optical rotatory dispersion, fluorescence, and deuterium exchange in state A.[6]

Our second conclusion then is that CGN is less completely folded in state A than CT. This conclusion is identical with that reached by Rupley, *et al.*,[7] but only accidentally since no information on differences in the degree of folding for CT and CGN is at present possible from O.R.D. or C.D. studies.[8,9] If crystal forces are inadequate to force complete folding, the unfolded parts "dissolved" in interstitial water have considerable freedom of segmental motion which will complicate analysis of X-ray diffraction intensities.

Transition I for monomethionine sulfoxide CT (MMSCT) and for CT have been found to be identical within error. Oxidation of methionine 179, which is farther in linear peptide distance from the acylatable serine to produce DMSCT, not only results in some unfolding in state A but alters catalytic rate parameters for some substrates. In agreement with Weiner, *et al.*,[10] we find small parameter changes with MMSCT on ester and amide substrates and with DMSCT on ester substrates, but these authors find at least a 500-fold reduction in hydrolysis rate using N-acetyl-L-tyrosinamide as substrate for DMSCT. The effects are probably not coincidental. The reduction in rate

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(2) J. F. Brandts, *J. Am. Chem. Soc.*, **86**, 4291 (1964).
(3) D. C. Poland and H. Scheraga, *Biopolymers*, **3**, 401 (1965).
(4) R. Lumry, R. Biltonen, and J. F. Brandts, *ibid.*, in press.
(5) J. F. Brandts, *J. Am. Chem. Soc.*, **86**, 4302 (1964).
(6) R. Biltonen, Dissertation, University of Minnesota, 1965.
(7) J. Rupley, W. Dreyer, and H. Neurath, *Biochim. Biophys. Acta*, **18**, 162 (1965).
(8) D. Ravel and J. Schellman, *ibid.*, in press.
(9) R. Biltonen, R. Lumry, V. Madison, and H. Parker, *Proc. Natl. Acad. Sci. U. S.*, in press.
(10) H. Weiner, C. W. Batt, and D. E. Koshland, Jr., *Federation Proc.*, 24, 473 (1965).

is then attributable to incomplete folding of DMSCT in state A. In the future more attention will have to be given to the possibility that specific chemical modification of single protein groups affects catalytic parameters indirectly through conformational alterations of this type. It is possible that the peculiar patterns of catalytic behavior distinguishing amide and peptide substrates from ester substrates, as, for example, is found with carboxypeptidases A[11] and B,[12] or large substrates from small substrates may be attributable to such changes in folding.

Complete details of the experiment and interpretation will be published soon.

(11) B. Vallee, J. Riordan, and J. Coleman. *Proc. Natl. Acad. Sci. U. S.*, **49**, 109 (1963).
(12) J. Folk, E. Wolff, E. Schirmer, and J. Cornfeld, *J. Biol. Chem.*, 237, 3105 (1962).

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Selective Chemical Modifications of Uridine and Pseudouridine in Polynucleotides and Their Effect on the Specificities of Ribonuclease and Phosphodiesterases

Sir:

1-Cyclohexyl-3-(2-morpholinyl-(4)-ethyl)carbodiimide metho-*p*-toluenesulfonate (CMC) has been shown to add specifically to uridine and guanosine components of ribonucleic acid (RNA) such that the modified uridine bases become resistant to the action of pancreatic ribonuclease.[1] As a result digestion of the modified RNA with this enzyme produces oligonucleotides which terminate with cytidine only.[2] It is now found that, when amino acid acceptor RNA from yeast is treated with CMC and hydrolyzed with ribonuclease, no pseudouridine phosphate, uridine phosphate, or their corresponding cyclic phosphates are produced. Thus it appears that pseudouridine is also blocked under the conditions necessary for the blocking of uridine and guanosine. Furthermore, if the modified RNA is treated with dilute ammonia to remove the blocking groups and then hydrolyzed with ribonuclease, the products obtained are similar to those obtained by the enzyme digestion of untreated RNA except that, in the former case, no pseudouridine phosphate or cyclic phosphate is formed. From these results it is implied that the pseudouridine bases in RNA form stable adducts with CMC and that the resistance of these adducts to ribonuclease hydrolysis results in the pseudouridine components being left in internal positions of the oligonucleotides that remain after the enzyme digestion.

These conclusions are confirmed by a study of the chemical blocking of pseudouridine itself. On reaction with CMC the nucleoside gives a mixture of two positively charged derivatives which can be separated by electrophoresis at pH 7. One of these has the electrophoretic mobility of a derivative containing one positive charge and is tentatively assigned the structure of the 3 adduct on the basis of its ultraviolet spectrum: λ_{max} 266 mμ at pH 7 and 292 mμ at pH 10. A structurally similar compound, 3-methyluracil, has λ_{max} 259 mμ at pH 7 and 282 mμ at pH 10, while 1-methyluracil has λ_{max} 268 mμ at pH 7 with no shift to longer wave lengths at higher pH values.[3] This assignment is supported by the fact that the 3 adduct is structurally analogous to the adduct obtained from uridine[1] and the fact that both of these adducts are resistant to ribonuclease when they are located in polynucleotide chains. The other derivative which must contain two positive charges in view of its electrophoretic mobility has λ_{max} 265 mμ at pH 7 and 9 (1,3-dimethyluracil has λ_{max} 266 mμ at these pH values[3]) and is assigned the structure of an adduct containing 2 molecules of CMC at the 1,3 positions. Its initial λ_{max} of 265 mμ at pH 9 changes to 292 mμ after 2 hr. at 25°, the shift presumably arising from the hydrolysis of the CMC group at the 1 position with the concomitant formation of a dissociable hydrogen atom. The 1,3 adduct can be converted to the 3 adduct by treatment with cold dilute ammonia, while the removal of the remaining blocking group from the latter requires hydrolysis with hot concentrated ammonia. Thus for sequence analysis studies it is now possible to selectively block guanosine, uridine, and pseudouridine in polynucleotides, imparting resistance to ribonuclease action at the positions occupied by the two pyrimidines. Further, by subsequent treatment with dilute ammonia a ribonuclease-resistant block can be produced at the pseudouridine positions only.

In order to study the effects of blocked nucleotides on the action of phosphodiesterases a number of dinucleoside phosphates were synthesized. A group of 2′,5′-diacetylnucleoside 3′-phosphates and 2′,3′-diacetylnucleosides were prepared, and appropriate combinations were condensed with dicyclohexylcarbodiimide by the method described by Rammler, *et al.*[4] The acetyl groups were then removed by dilute ammonia treatment and portions of the dinucleoside phosphates were treated with CMC under conditions previously described.[1] In the case of dinucleoside phosphates containing pseudouridine the products from the CMC reaction were subsequently treated with dilute ammonia to leave the pseudouridine moieties with one blocking group.

Table I lists various dinucleoside phosphates and their blocked derivatives and shows a comparison of their rates of hydrolysis with snake venom and spleen diesterases.[5] Cp$\bar{\psi}$ (cytidylyl-(3′→5′)-pseudouridine containing a blocking group on the pseudouridine) and Cp$\bar{\mathrm{U}}$ are both resistant to both of these enzymes. Thus it appears that a chemical block at uridine or pseudouridine in a polynucleotide chain will halt the normal hydrolytic progression of these exonucleases along the chain, a technique which is expected to be of some value in the determination of base sequences of nucleic acids. In addition, as mentioned above, $\bar{\mathrm{U}}$pA, $\bar{\psi}$pA, and $\bar{\mathrm{U}}$pC are resistant to hydrolysis by pancreatic ribonuclease.

The results listed in Table I also show an interesting difference in the effects of blocking groups on the actions

(1) P. T. Gilham, *J. Am. Chem. Soc.*, **84**, 687 (1962).
(2) J. C. Lee, N. W. Y. Ho, and P. T. Gilham, *Biochim. Biophys. Acta*, **95**, 503 (1965).
(3) D. Shugar and J. J. Fox, *ibid.*, **9**, 199 (1952).
(4) D. H. Rammler, Y. Lapidot, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 1989 (1963).
(5) Worthington Biochemical Corporation, Freehold, N. J.