* Present address: Department of Botany, University of Illinois, Urbana, Illinois.
† Contribution no. 68.

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USE OF "REPORTER GROUPS" IN STRUCTURE-FUNCTION STUDIES OF PROTEINS*

BY MERRILL BURR AND D. E. KOSHLAND, JR.

THE ROCKEFELLER INSTITUTE, AND THE BIOLOGY DEPARTMENT OF BROOKHAVEN NATIONAL LABORATORY

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Correlation of structure and function in protein molecules has taken enormous strides in recent years. X-ray crystallography has made it possible to delineate the three-dimensional structure of hemoglobin and myoglobin,[1, 2] and protein modification methods have identified amino acids at the active sites of a number of enzymes.[3–5] It would appear that these techniques can be applied in other cases and thus both the three-dimensional structures of many proteins and the positions in these structures of essential amino acids will be known. It is, therefore, not premature to ask what further information will be needed to complete the correlation of function and structure and whether tools can be devised to provide this information.

One problem which remains, even after the active site has been located, is the description of the steric relations between the various residues at the active site and the various parts of the substrate molecule. This problem is illustrated in Figure 1. If E represents an essential residue, e.g., the serine in chymotrypsin, it seems clear that part of the substrate must be in contact with this residue but the alignment of other parts of the substrate is not determined. Thus, a requirement that the amino

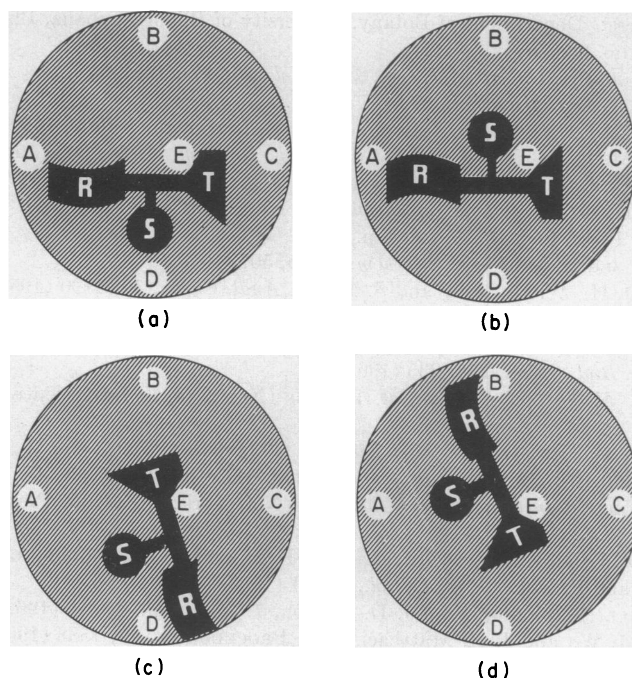

FIG. 1.—Orientations of substrate on a hypothetical enzyme surface. A, B, C, D, E represent positions of amino acid residues. All orientations place an essential residue E adjacent to the T moiety of the substrate. The position of the substrate in (a) and (b) are achieved by a 180° out-of-plane rotation, whereas in (d) the position is achieved by movement of the substrate only within the plane of the enzyme surface.

acid residue E of Figure 1 must be near the T moiety of the substrate still leaves a number of alternatives for the location of the R and S portions of the substrate.

A second problem which is related to the first, but which poses separate experimental and theoretical difficulties arises from the need to determine the contributions to binding and catalysis made by the interaction of specific parts of the substrate with specific parts of the protein. The successful solution of the first problem, e.g., the finding that the R moiety of the given substrate is adjacent to the amino acid residue, B, in the ES complex will be a giant step, but it will not establish the contribution of this interaction unless R or B, or both, can be modified and the binding and velocity constants for the new modifications correlated with the original values. To make such a comparison the orientations of the new substrates and inhibitors on the protein surface must be known, and it cannot be presumed *a priori* that their orientation will be analogous to the original system. Niemann and co-workers[6] have used the concept of variable orientation of substrates in an attempt to correlate the kinetic constants of a wide variety of chymotrypsin substrates. Also, the substrate for β-amylase has been shown to act as its own competitive inhibitor by binding incorrectly to the active site.[7]

The logical consequence of such considerations can be illustrated by reference to Figure 1. A given substrate, for example, might be bound to an enzyme in the orientation of Figure 1a. Modification of either the S group of this substrate or the D group of the protein might produce a new alignment similar to that of

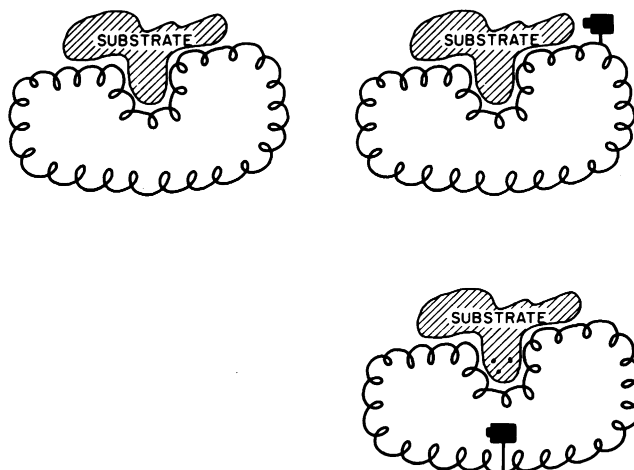

FIG. 2.—Schematic representation of enzyme-substrate complex in native protein, protein containing reporter group (solid black area) adjacent to substrate binding area and reporter group distant from substrate binding area.

Figure 1*b*. Interpretation of kinetic constants for the modified system would, therefore, be incorrect if the orientation of Figure 1*a* was assumed throughout. Because of this alternative a ready and objective criterion will be needed to establish orientations in modified systems relative to a standard system.

A third problem relates to the role of flexibility in enzyme action. It seems increasingly apparent that conformation changes play a vital role in the specificity process[8] and these changes make the structure-function correlation more complex. For example, it will be important to know which parts of the protein undergo conformational changes on interaction with the substrate. Moreover, the flexibility hypothesis[9] assumes that the alignment of catalytic groups is involved and, thus, conformation changes may be identified not only with the initial binding process, but also with the subsequent catalytic steps. It will be desirable to identify the conformational changes with the appropriate steps in the enzyme action.

To aid the solution of these and related problems, a new technique has been developed based on the rationale of Figure 2. Briefly stated, it involves the introduction of an environmentally sensitive residue into specific positions of the protein molecule. The environmentally sensitive group will be referred to as a "reporter" group since it is designed to "report" changes in its environment to an appropriate detector. Two situations are shown in which a reporter group might register environmental changes. In one, the reporter moiety is placed next to the active site so that a direct interaction with substrate is possible. In the other, the reporter group is placed at a point distant from the active site. In the former case the absorption of the substrate or inhibitor[10] to the active site changes the environment of the reporter group by direct contact. In the latter case, direct contact with substrate is excluded but a substrate-induced conformation change may trigger changes in conformation in the neighborhood of the reporter group.

To be of maximum utility, the position occupied by the reporter group must be known, and the simplest method of achieving this is to attach it by a covalent bond

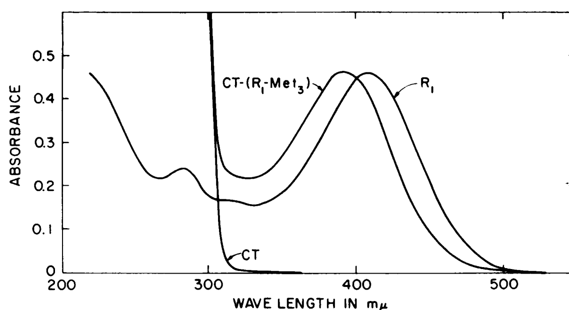

FIG. 3.—Spectra of chymotrypsin (CT), 2-acetamido-4-nitrophenol (R_1), and chymotrypsin which has been reacted with 2-bromoacetamido-4-nitrophenol. The nomenclature CT-(R_1-Met_3) reflects the fact that the reporter group (R_1) is attached to the methionine three residues from the active serine of the chymotrypsin.

to a specific residue in the protein. The original reagent must, therefore, contain both a "reporter moiety," which is sensitive to changes in environment, and a "positioning" group which will guide and bond the reporter moiety to the appropriate residue in the protein. One such reagent, 2-hydroxy-5-nitrobenzyl bromide, has already been described.[11] In this paper a second reagent is described, together with some preliminary experiments which indicate that this approach will be of general utility in the problems outlined above.

The reagent, 2-bromoacetamido-4-nitrophenol (I), was designed based on the knowledge (*a*) that the nitrophenol chromophoric group is highly sensitive to its environment and absorbs in a region of the spectrum in which proteins are transparent, (*b*) that chymotrypsin contains a methionine residue which is three residues from the active serine in the primary sequence,[12] (*c*) that this methionine residue can be modified by iodoacetamide without modification of any other residue in the protein,[13] and (*d*) that this methionine can be carboxymethylated with only minor changes in the activity of the enzyme.[13]

Treatment of 2-amino-4-nitrophenol with bromoacetyl bromide in acetone by classical procedures[14] produced the compound which was crystallized from ethanol and found to melt with decomposition at 212–213°. NMR and infrared spectra were consistent with this structural formula as was the carbon-hydrogen analysis. On treatment of 8×10^{-5} *M* chymotrypsin for 18 days in 20 per cent methanol at pH 3, 20° with 9×10^{-4} *M* compound I, 0.6 moles of reporter group were introduced per mole of chymotrypsin. The spectra of the free reagent, of the native chymotrypsin, and of the labeled protein are shown in Figure 3. The enzyme has 40 per cent of its native activity as tested by the method of Schwert and Takenaka.[15]

Difference spectra were obtained by comparing solutions of the labeled enzyme in the presence of substrate and buffer with solutions of labeled enzyme in the presence of buffer only. Although this reporter group is sensitive to pH (a fact which can be used to advantage in some studies), this property was not of interest in the present studies. The solutions were carefully buffered and pH measurements were made before and after mixing of substrate with enzyme to ensure that pH changes were less than 0.01 pH units. In Figure 4*e*, the difference spectrum obtained with a change in pH of 0.01 pH units is shown to indicate the spectrum to be expected from such a source.

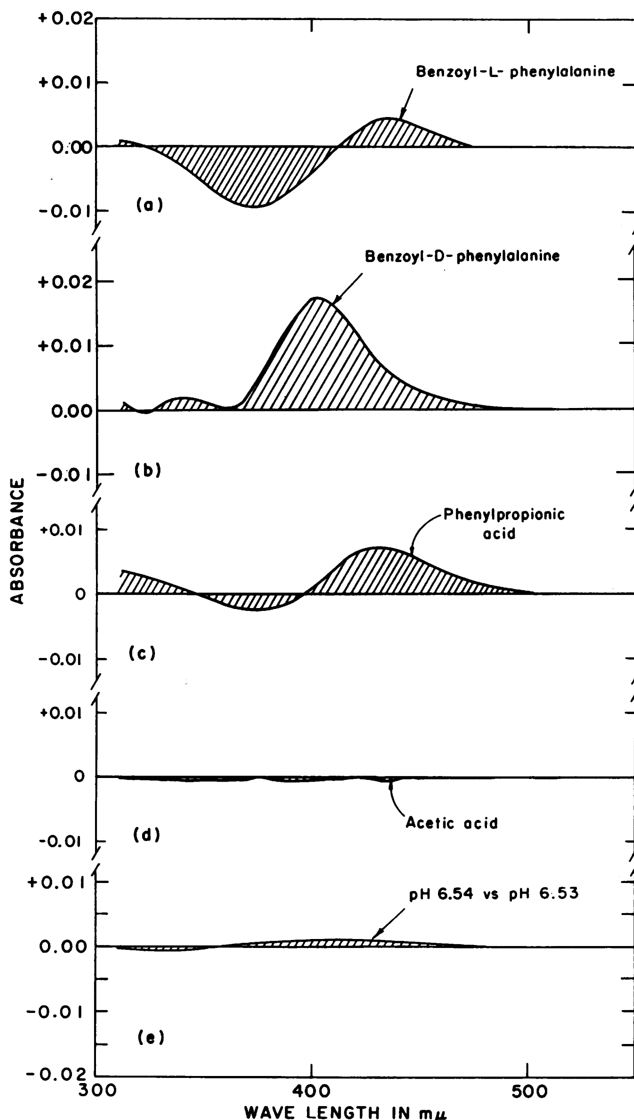

FIG. 4.—Difference spectra of chymotrypsin labeled with a reporter group CT-(R_1-Met_3). Conditions: 7.4×10^{-5} *M* enzyme, 0.05 *M* phosphate buffer; (*a*)–(*d*) 1.5×10^{-3} *M* substrate, pH 6.49; (*e*) spectrum calculated for pH change of 0.01 pH units based on measured spectra for a change of 0.10 and 0.02 pH units.

Some representative spectra shown in Figure 4 establish that significant difference spectra are obtained in the presence of some substrates of chymotrypsin. In each case the free acid was used (substrate for the back reaction) to obviate pH errors resulting from hydrolysis. Controls with free dyes and substrates showed no difference spectra. A control with acetic acid (Fig. 4*d*) showed essentially no difference spectrum indicating that ionic strength effects or a nonspecific carboxyl interaction were not responsible for the difference spectra of Fig. 4*a–c*. Benzoyl phenylalanine, the ethyl ester of which is an excellent substrate for the enzyme, gives

pronounced difference spectra, those of the D and L isomers being significantly different from each other. Acetyl-L-phenylalanine and benzoyl-L-alanine give similar but less pronounced differences. Considering even these few examples, it is clear that the spectrum is a function of a specific interaction of substrate and enzyme. Thus, the first desired goal has been achieved—an environmentally sensitive group has been introduced in a sensitive region of the protein and has been shown to report changes which are related to specific structural features of the substrate.

Even from these early studies some of the ramifications and limitations of this method are indicated. They are as follows:

(1) A relative correlation of substrate structure and orientation of the protein may be obtained without knowledge of the three-dimensional structure of the protein. Moreover, the approximate relative positions of a residue of the protein and a portion of the substrate may be obtained. Using Figure 1 as an illustrative example, all substrates with a bulky *R* group might cause a signal change in a reporter group attached to residue *A* on the protein, whereas substrates with a bulky *T* group might interact with a reporter group attached to residue *C*. Thus, new specificity information will become available even if X-ray data are not completed, and the specificity information will become more precise and meaningful, rather than become obsolete, when the three-dimensional structure of the protein is known. It should be emphasized that the ease with which orientations can be assigned to substrates is not yet known.

(2) Either a direct interaction with substrate or an indirect conformation change can cause a signal from the reporter group. Hence the observation of such a signal does not *a priori* determine which type of change is occurring. Knowledge of the structure of the protein as revealed by X-ray data may resolve this dilemma, e.g., the reporter group may be positioned so far from the active site that direct interaction is impossible. In the absence of such information, techniques now in existence for detecting conformational changes in solution may be used.[8]

(3) A reporter group should be particularly useful in stopped flow and temperature-jump experiments. By focusing on the reporter group, direct interactions and conformational changes may be correlated with individual steps in the enzymatic reaction.

(4) A reporter moiety can be attached either to an essential or to a nonessential residue. Perhaps the maximum information will be obtained by attaching the reporter group to a residue which is sensitive to changes at the active site but which has only a minor effect on the enzyme action, such as the methionine in chymotrypsin. The active enzyme can, in this case, be examined during essentially normal functioning. However, it is possible that modification of an essential catalytic group may occur without, for example, change in the binding process. In this case the reporter moiety could be used to study the binding phenomenon without the complication of the simultaneous catalytic process.

(5) The positioning group of the reagent does not have to have absolute specificity, but its effectiveness will depend in part on fairly high specificity and in part on the characteristics of the individual protein under study. For example, previous work had indicated that an iodoacetamide derivative would attack only the surface methionine residue in chymotrypsin under acid conditions and this appears to be

true also for 2-bromoacetamido-4-nitrophenol. This reagent should also attack sulfhydryl residues in proteins containing cysteine, and preliminary experiments with glyceraldehyde phosphate dehydrogenase indicate that such reaction occurs. Since the SH reactivity is greater than that of a thioether, the reagent may even be specific for SH in the presence of methionine under proper experimental condition.

If reaction with several residues occurs, chromatography may be able to separate the monosubstitution products as has been done in the cases of derivatives of ribonuclease by Crestfield, Stein, and Moore,[5] and Hirs.[4]

Moreover, "positioning groups" of different specificity can guide the "reporter" group to different protein sites. The previously reported reagent, for example, was specific for tryptophan but contained the same reporter group. By judicious choice of reagents the regions of the protein which are affected or unchanged by substrate may be "mapped out."

(6) Permutations on the same principle will allow application to many problems, e.g., antibody-antigen interactions, association of subunits, etc. Moreover, different types of reporter groups are possible, e.g., fluorescing groups or groups generating electron spin resonance signals.

Summary.—A new technique has been developed for the correlation of function with structure in protein molecules. A group which is sensitive to changes in environment and which can transmit a signal to an appropriate detector is introduced into a specific position in the protein. This group may report changes in its environment caused either by direct interaction with substrate or by a conformational alteration in the protein structure. An application of this method is described in which the compound 2-bromoacetamido-4-nitrophenol is reacted with the methionine residue near the active serine of chymotrypsin. On mixing substrates and inhibitors with the modified protein, characteristic difference spectra are obtained. Some of the applications and limitations of the method are discussed.

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[10] Clearly, most of the statements in which substrates are discussed are equally true for inhibitors, haptens, etc., but for clarity the phrase "and inhibitor" will not be included each time.

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THE DYNAMICS OF THE MEMBRANE-BOUND INCOMPRESSIBLE BODY: A MECHANISM OF CELLULAR AND SUBCELLULAR MOTILITY*

BY PAUL WEISS

THE ROCKEFELLER INSTITUTE

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Let us start from the widely accepted Danielli-Davson model of the cell surface as a composite sheet consisting of transversally oriented lipid molecules on the outside, lined by a tangentially oriented network of protein chains underneath. If we view this smectic system not in its customary schematized representation as an open fragment with a free edge, but in its natural configuration of a closed system, it reveals dynamic properties of great biological significance not formerly recognized. The real structure differs from its schematic sample in two regards: (1) it is a *continuous* two-dimensional body, completely closed up within itself; and (2) it envelops a closed space filled with substance of high water content, hence, very low compressibility (to be referred to in the following as "core").

The properties emerging from this configuration rest on the following premises: (*a*) The average population density of the molecules in the lipid "picket fence" is determined by the equilibrium between the cohesion due to van der Waals' forces and the dispersive pressure exerted from the enclosed core. (*b*) The surface membrane constitutes a permeability barrier between the outside medium and the core. (*c*) The permeability of the surface layers increases with increasing intermolecular distances: widening of intermolecular "pores" renders the membrane "leaky." Assuming water in the core to be mostly in bound and polymerized form, the increased flux pertains chiefly to ions and small molecules. (*d*) The lipid layer is "spot-welded" to the polar side chains of the underlying protein layer by electrostatic bonds, so that any local deformation of either layer is mechanically transmitted to the other. (*e*) There is potential energy ("strain") stored within the system which upon local release can effect contraction of the protein net; by changing the array and packing density of the molecules of the surface membrane, this contraction engenders local deformations. (*f*) Increased flux of ions and molecules across the membrane according to (*c*) can activate the energy release cited in (*e*).

Let us now consider a cylindrical core of fluid or semifluid substance of negligible compressibility, completely enveloped by a membrane of the listed properties. Let then one end (hereafter called "proximal") of this cylinder become the site of a chemical, electrical, or mechanical alteration that causes the molecules of the sur-