Chakraborty, K. P., and Hurlbert, R. B. (1961), *Biochim. Biophys. Acta 47*, 607.
Duffield, R. B., and Calvin, M. (1946), *J. Amer. Chem. Soc. 68*, 557.
Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.
Hartman, S. C. (1963), *J. Biol. Chem. 238*, 3024.
Khedouri, E., Anderson, P. M., and Meister, A. (1966), *Biochemistry 5*, 3552.
Levitzki, A. (1970), *Anal. Biochem. 33*, 335.
Levitzki, A., and Koshland, D. E., Jr. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 1121.
Levitzki, A., and Koshland, D. E., Jr. (1970), *Biochim. Biophys. Acta 306*, 473.
Levitzki, A., Stallcup, W. B., and Koshland, D. E., Jr. (1971), *Biochemistry 10*, 3371.
Lieberman, I. (1955), *J. Amer. Chem. Soc. 77*, 2661.
Lieberman, I. (1956), *J. Biol. Chem. 222*, 765.
Long, C. W., Levitzki, A., and Koshland, D. E., Jr. (1970), *J. Biol. Chem. 245*, 80.
Long, C. W., and Pardee, A. B. (1967), *J. Biol. Chem. 242*, 4715.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Mizobuchi, K., Kenyon, G. L., and Buchanan, J. M. (1968), *J. Biol. Chem. 243*, 4863.
Moos, C., Alpert, N. R., and Myers, T. C. (1960), *Arch. Biochem. Biophys. 88*, 183.
Pamiljans, V., Krishnaswamy, P. R., Dumville, G., and Meister, A. (1962), *Biochemistry 1*, 153.
Randerath, K. (1964), *J. Chromatogr. 16*, 111.
Sartorelli, L., Fromm, H. J., Benson, R. W., and Boyer, P. D. (1966), *Biochemistry 5*, 2877.
Wahl, A. C., and Bonner, N. A. (1951), Radioactivity Applied to Chemistry, New York, N. Y., Wiley, p 7.
Yount, R. G., Babcock, D., Ballantyne, W., and Ojata, D. (1971), *Biochemistry 10*, 2484.

Half-of-the-Sites Reactivity and the Conformational States of Cytidine Triphosphate Synthetase*

Alexander Levitzki,† William B. Stallcup, and D. E. Koshland, Jr.‡

ABSTRACT: The affinity label 6-diazo-5-oxonorleucine (DON) reacts with only half of the total glutamine sites of CTP-synthetase, even though the subunits are identical. The binding of DON to half of the subunits abolishes the glutamine activity of the enzyme; thus, vacant active sites as well as occupied sites are turned off. However, the DON-labeled enzyme retains its overall activity with ammonia and its characteristic binding of other ligands such as ATP, UTP, and GTP. It is postulated that DON labeling is an example of extreme or absolute negative cooperativity in which the conformational change induced by DON in the covalently labeled subunit is transmitted to the neighboring subunit so that no DON reaction can occur there. This model provides a general explanation for other enzymes in which half-of-the-sites reactivity has been observed. The fact that the DON-induced change can abolish glutamine activity while leaving ammonia activity and nucleotide binding unaffected indicates that the protein is designed to allow specific transmission of conformational changes.

An increasing number of enzymes have been shown to have "half-of-the-sites reactivity." By this we mean that these proteins are composed of identical subunits with n potential sites and yet they react with a substrate or an inhibitor so that only $n/2$ sites are occupied when the enzyme is saturated with that ligand. A summary of enzymes which exhibit this phenomenon is shown in Table I. The phenomenon seems to originate from the architecture of the multisubunit enzymes exhibiting the property and therefore its analysis may reveal clues to protein design.

CTP-synthetase[1] is a particularly attractive example with which to pursue this problem, since the chemistry of its interactions has been elucidated (Levitzki and Koshland, 1971) and since it was shown to exhibit an apparent half-of-the-sites reactivity with the affinity label, 6-diazo-5-oxonorleucine (DON) (Long *et al.*, 1970). Accordingly, the reactivity of DON with CTP-synthetase in the dimer and tetramer forms and the reactivity of this affinity label with the protein containing varying amounts of substrates and activators were studied. The results not only clarify the nature of half-of-the-sites reactivity, but reveal an interesting mosaic of interactions between the different ligand sites within a single subunit and between different subunits.

Materials and Methods

CTP-synthetase was prepared and assayed as described earlier (Long *et al.*, 1970; Levitzki and Koshland, 1970). DON was obtained from Parke-Davis. Chemicals were of the

* From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received April 13, 1971.* The authors acknowledge the support of the National Science Foundation (Grant GB-7057) and the U. S. Public Health Services (Grant AM-GM-09765). W. B. S. is the recipient of the Smith, Kline & French Laboratories Fellowship.

† Present address: Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel.
‡ To whom correspondence should be addressed.

[1] Abbreviations used are: CTP, cytidine 5'-triphosphate; DON, 6-diazo-5-oxonorleucine; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); CTPS, CTP-synthetase; F_2DPS, *p,p'*-difluoro-*m,m'*-dinitrophenylsulfone.

TABLE I: Half-of-the-Sites Reactivity in Multisubunit Enzymes.

Enzyme	No. of Subunits	Ligand Tested	No. of Binding Sites	Reference
Alcohol dehydrogenase	2	NADH, alcohols[a]	1.0[b]	Bernhard *et al.* (1970)
UDP-Gal-epimerase	2	NAD[a]	1.0	Wilson and Hogness (1964, 1969)
Alkaline phosphatase	2	2,4-DNP-phosphate	1.0	Trentham and Gutfreund (1968)
	2	P_i[a]	1.0	Detitdems *et al.* (1970) and Simpson and Vallee (1970)
Glutamine-PRPP-amido-transferase	2	DON[c]	1.0	Hartman (1963) and Rowe and Wyngaarden (1968)
CTP-synthetase	2	DON[c]	1.0	
CTP-synthetase	4	DON[c]	2.0	
Glyceraldehyde-3-P dehydrogenase from yeast	4	F_2DPS[c]	2.0	Givol (1969)
Glyceraldehyde-3-P dehydrogenase from rabbit muscle	4	β-(2-Furyl)acryloyl phosphate	2.0	Malhotra and Bernhard (1968)
Malate dehydrogenase	2	Ketomalonate	1.0	Harada and Wolfe (1968)
Acetoacetate decarboxylase	8	Acetic anhydride[c]	4.0	O'Leary and Westheimer (1968)
	8	2,4-DNP-acetate[c]	4.0	
Glutamine synthetase	8	Methionine sulfoxamine	4.0	Tate and Meister (1971)

[a] Substrate. [b] H. d'A. Heck, personal communication. [c] Affinity label.

highest purity available. Procedures, unless discussed in detail, were conventional in nature and similar to those described in previous papers (Long *et al.*, 1970; Levitzki and Koshland, 1971).

Results

Kinetics of DON Reaction with CTP-synthetase. The rate of reaction of DON with CTP-synthetase is shown in Figure 1, and the rate constants calculated from these data are summarized in Table II. The results in Table II illustrate the complexity of the reaction and the manner in which added ligands or the state of aggregation of the enzyme can affect the reactivity towards DON. In all cases, even after long intervals of time only half of the total subunits are labeled with the affinity label which so closely resembles glutamine (Table III). In the absence of any added ligands the protein exists as a dimer (Long *et al.*, 1970) and since only one site per dimer reacts, a single rate constant is observed.

When GTP at saturating concentrations is added to the dimer, the DON still reacts with only one of the two subunits but apparently the GTP induces a conformational change such that the rate of reaction of the DON is now increased by approximately eightfold. Because DON is an affinity label mimicking the structure of glutamine, this increase is logical since GTP activates the glutamine reaction of the CTP-synthetase.

When ATP and UTP at saturating concentrations are added to the enzyme, the dimer polymerizes to a tetramer of molecular weight 216,000 (Long *et al.*, 1970; Levitzki and Koshland, 1970). DON reacts with the tetramer so that two of the four sites become covalently labeled. In this case, the rate constants for the first and second sites are different. The first of these sites shows a rate constant even faster than that for the dimer saturated with GTP. The reaction of the first DON must induce a conformational change with a negatively cooperative effect because the second molecule of DON reacts $^1/_{25}$ as rapidly as the first.

If GTP is added in addition to UTP and ATP the effect on a tetramer is different from the effect on the dimer. The reactivity of the first DON is about equal to that of the GTP-saturated dimer but GTP does abolish the negative cooperative effect. Thus, only one rate constant of 3.5×10^3 min^{-1} is observed, slower than the reaction rate of the first molecule of DON in the absence of GTP.

Properties of the DON Enzyme. Since the DON-modified enzyme possesses no glutamine activity, but possesses normal ammonia activity, the K_m and k_{cat} values for ammonia can be studied on this modified enzyme and compared with those of the native. The kinetic parameters are summarized in Table IV. It can easily be seen that both the Hill coefficient and $s_{0.5}$ values for ATP and UTP are very similar in the native and modified enzymes. Binding measurements using [^{14}C]UTP and [^{14}C]ATP also revealed that the native enzyme and the DON-enzyme gave the same binding curves. A typical example is given in Figure 2. Thus, in spite of the fact that the glutamine site must be immediately adjacent to the ammonia site and hence in close physical proximity to the ATP and UTP sites, the DON reaction has essentially no effect on the kinetics of the reaction with ammonia and no effect on the binding characteristics of UTP and ATP.

Polyacrylamide Gel Electrophoresis and Electrofocusing. Polyacrylamide gel electrophoresis was performed at pH 9.5 and 8.0 in 8 M urea. CTP-synthetase yielded a single band in each case. The conditions in these experiments were identical with those which showed separation of yeast glyceraldehyde 3-phosphate dehydrogenase isozymes and in which high discrimination was observed in other isozyme situations. The

TABLE II: Effect of Ligands on Rate of DON Inactivation.[a]

Ligands	Mol Species of CTPS	k_{DON} (min^{-1})
Mg^{2+}	105,000 (dimer)	0.52×10^3
GTP + Mg^{2+}	105,000 (dimer)	4.0×10^3
ATP + UTP + Mg^{2+}	210,000 (tetramer)	$k_1 = 9.7 \times 10^{3}$[b]
		$k_2 = 0.35 \times 10^3$
ATP + UTP + GTP + Mg^{2+}	210,000 (tetramer)	3.53×10^3

[a] Enzyme (2.6 units, 7×10^{-6} M in sites) was inactivated with 8.9×10^{-5} M DON in the presence of 0.02 M imidazole acetate (pH 7.20) at 0°. $k_{DON} = k_{obsd}$/[DON]. Different ligands were present as is indicated in the table. [Mg^{2+}] = 0.01 M, [GTP] = 2.1×10^{-3} M, [ATP] = 1.6×10^{-3} M, [UTP] = 1.6×10^{-3} M. Aliquots were removed at different times to check both glutamine and ammonia activity. [b] Rate calculated after subtracting limiting slope of less reactive residues in Figure 1.

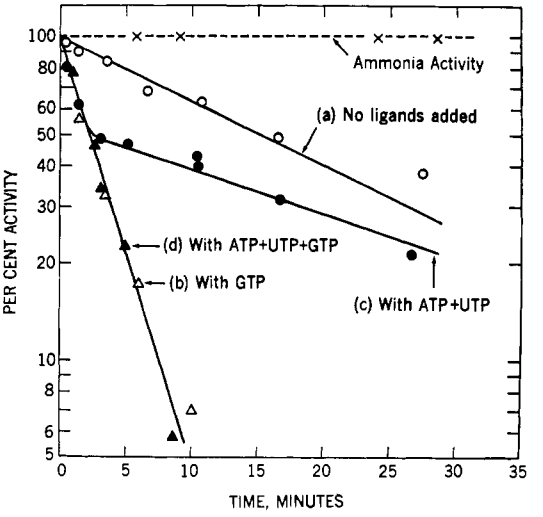

FIGURE 1: The effect of GTP and state of aggregation on the rate of DON inactivation of CTP-synthetase. CTP-synthetase (0.257 unit of specific activity = 6.1 units/mg, 3.47×10^{-6} M in monomer subunits) was incubated with various ligands in the presence of 0.02 M imidazole acetate (pH 7.2), 0.01 M $MgCl_2$, and 8.9×10^{-5} M DON at 0°. Curves recorded for: (a) no ligands added (enzyme present as dimer); (b) 2.1×10^{-3} M GTP (enzyme present as dimer); (c) 1.6×10^{-3} M UTP and 1.6×10^{-3} M ATP (enzyme present as tetramer); and (d) 1.6×10^{-3} M UTP, 1.6×10^{-3} M ATP, and 2.1×10^{-3} M GTP (enzyme present as tetramer).

molecular weight of CTPS under these conditions was shown to be that of the monomer (50,000) (Figure 3A).

Gel electrofocusing in 8 M urea (Wrigley, 1968) again gave a single band for CTPS. The DON-CTPS also gave a single band under these conditions but this band is distinct from that of the unmodified CTPS. This result was extremely puzzling. First of all, it is surprising that the technique is sensitive enough to distinguish such a minor change as the DON label. At the isoelectric point of 5.5 this label adds no net charge to the protein. Secondly, if the DON-labeled protein is separable from unlabeled protein, then the DON-labeled enzyme should yield two bands since only half of the subunits are labeled. The anomaly was resolved by the finding that in 8 M urea at the isoelectric point of 5.5 or any fairly low pH such as 4.0, the dimer does not dissociate to monomers (Figure 3B). A mixture of 5 M urea and 2 M *N*-butylurea was also unable to dissociate the dimer at low pH. The fully carboxymethylated enzyme also does not dissociate at low pH values whereas it does dissociate in 8 M urea at pH 7.2 (Long *et al.*, 1970).

The gel electrophoresis at pH 8.0 (conditions under which dissociation does occur) does not separate DON-labeled subunits from unlabeled ones. It will separate carboxymethylated subunits from native subunits (five charges) very well. We considered this circumstantial evidence that the chains are identical although a subtle difference might go undetected by this technique. The improbability of isozymes can be supported by the electrofocusing technique—since a three-band pattern

TABLE III: Effect of Ligands and State of Aggregation on Binding of DON and Glutamine to CTP-synthetase.

Ligands	Enzyme Species	Max. No. of Sites: Glutamine	Max. No. of Sites: DON
None	105,000 (dimer)	2.05	0.86–1.03
ATP + UTP + GTP	210,000 (tetramer)	*a*	2.10
DON-CTP-synthetase	Dimer	<0.01	1.0 ± 0.1
Enzyme + [^{14}C]-glutamine[b]		<0.01	1.0 ± 0.1

[a] Not measured because of rapid glutamine turnover. The reaction with [^{14}C]DON was performed as described earlier (Long *et al.*, 1970). [b] In this experiment the enzyme dimer was equilibrated with [^{14}C]glutamine before the addition of nonlabeled DON. As is seen in the table, no glutamine was "trapped" at the site which does not react with DON.

TABLE IV: Cooperativity Parameters for DON-CTP-synthetase and the Native Enzyme.[a]

	Hill Coef: CTPS	Hill Coef: DON-CTPS	$s_{0.5}$ (mM): CTPS	$s_{0.5}$ (mM): DON-CTPS
ATP	2.87	2.85	0.43	0.45
UTP[b]	1.75	1.85	0.39	0.37
UTP[c]	1.10	1.0	0.40	0.40
NH_3	1.0	1.0	5.3	7.0

[a] Assay solutions contained the standard assay mixture: 0.025 M $(NH_4)_2SO_4$, 0.02 M Tris-acetate buffer (pH 8.15), 0.01 M $MgCl_2$, UTP, and ATP as specified in the table, in a final volume of 1.0 ml. The assay was performed at 38° (Long *et al.*, 1970). [b] [ATP] = 3.75×10^{-4} M (subsaturating). [c] [ATP] = 8.5×10^{-4} M (saturating).

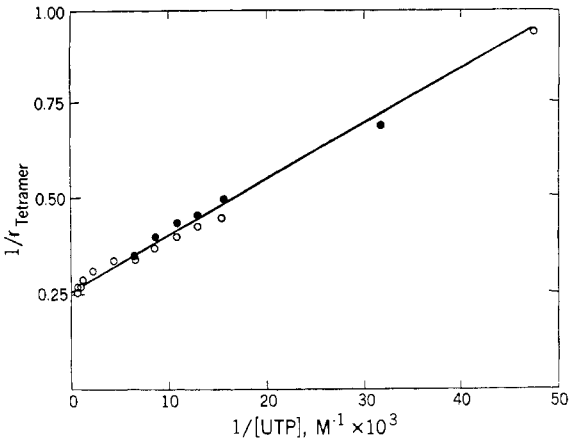

FIGURE 2: Binding of [^{14}C]UTP to native CTP-synthetase and DON-enzyme. Binding of [^{14}C]UTP was measured in the presence of 1.5 $\times$ 10^{-3} M ATP, 0.01 M $MgCl_2$, 0.01 M imidazole acetate (pH 7.2), 0.008 M sodium phosphate (pH 7.4), 0.030 mM β-mercaptoethanol, 2 $\times$ 10^{-3} M L-glutamine, and 5 $\times$ 10^{-4} M EDTA. The tetramer concentration in the compartment was 0.44 $\times$ 10^{-6} M. The DON enzyme was prepared by adding 60 μmoles of DON/1.4 units of enzyme at 0°. (○) Native CTP-synthetase; (●) DON-enzyme.

was never seen. If there are nonidentical subunits, the differences between them must be small or there must be a preferred $\alpha\beta$-type interaction such that $\alpha\alpha$ and $\beta\beta$ do not occur.

Reactivity of Protein SH Groups with DTNB. The stoichiometry of the reaction of DTNB with CTP-synthetase is summarized in Table V. In urea, ten free sulfhydryl groups are found in the dimer and nine in the DON-treated CTP-synthetase dimer, thus substantiating the results obtained with iodoacetic acid (Long *et al.*, 1970).

TABLE V: Reaction of DTNB with CTP-synthetase.[a]

Enzyme Prepn	No. of SH Groups Titrated in 8 M Urea/ 105,000	No. of SH Groups Titrated in Absence of Urea	$t_{1/2}$ of Slowest SH Groups in Native State (min)
Deglutaminated CTPS	9.9	10.2	1.3
Glutamine-CTPS	10.1	10.1	6.1
DON-CTPS	8.9	9.1	72

[a] CTPS freed of glutamine and β-mercaptoethanol on a G-25 column was treated with the indicated ligands and then with a 100-fold excess of DTNB. Absorbance was followed at 412 mμ at pH 8.0. ϵ_M = 13,600 (Ellman, 1959).

Distinct differences between the three forms of the enzyme (native, glutamylated, and DON) can be demonstrated by following the kinetics of the reaction with DTNB (*cf.* Figure 4). DON and glutamine affect the reactivities of several SH groups and affect them in different ways. The glutamylated enzyme has five or six SH groups which react almost five times more slowly than the corresponding groups in the deglutamylated enzyme. DON-labeled enzyme seems to have some SH groups of intermediate reactivity and about 3 groups which react more than 10 times less rapidly than any of the groups of the glutamylated enzyme and more than 50 times slower than those of the deglutamylated enzyme. These studies indicate that DON causes a major change in the conformation of the protein and is consistent with the conclusion that the DON effect is more drastic than glutamylation.

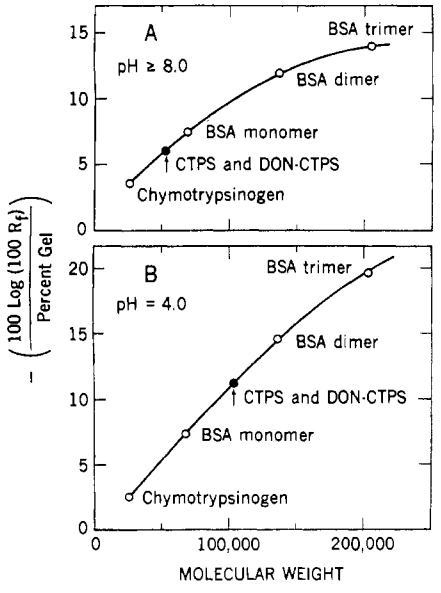

FIGURE 3: The molecular weight of CTP-synthetase and DON-CTP-synthetase in 8 M urea at pH 9.5 or 8.0 (A) and 4.0 (B). The method used is that of Hedrick and Smith (1968), the only difference being the inclusion of urea in the gels. 5, 7.5, and 10% gels were used to determine the slope = (100 log (100R_F))/% gel. Bovine serum albumin and chymotrypsinogen were run as standards. Bromophenol blue was the marker in the case of the pH 9.5 and 8.0 runs. Neutral red was used for the pH 4.0 runs. The pH 9.5 system is that of Davis (1964) while the pH 4.0 system is that of Reisfeld *et al.* (1962). The pH 8.0 system is a new one designed according to the principles of Williams and Reisfeld (1964). It employs chloride as the leading ion, bicine as the trailing ion, and imidazole as the buffer. The running buffer is adjusted to pH 7.5, the starting buffer to pH 6.0, and the electrode buffer to pH 7.1.

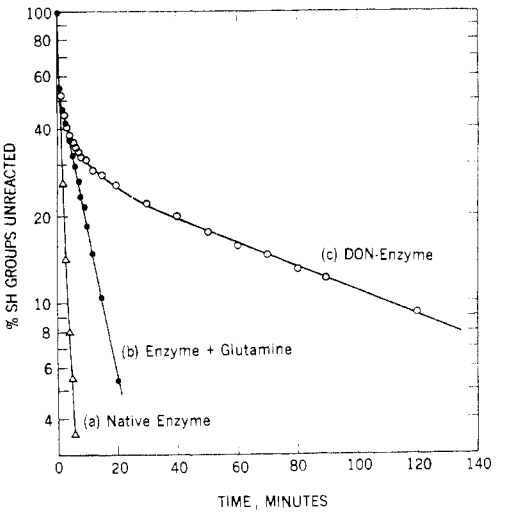

FIGURE 4: Semilog plot of reaction of DTNB with CTP-synthetase, DON-CTP-synthetase and glutamyl CTP-synthetase. CTP-synthetase freed of β-mercaptoethanol and all ligands on a G-25 column was treated as indicated in each case and then treated with a 100-fold excess of DTNB. OD was followed at 412 mμ at pH 8.0. ϵ_M 13,600 (Ellman, 1959).

Whatever the mechanism of the DON effect, it is clear that both DON and glutamine react with the enzyme to induce major conformational changes in the protein molecule.

Discussion

Half-of-the-Sites Reactivity. A significant number of enzymes have now been shown to exhibit the paradox of half-of-the-sites reactivity, in which only one of two apparently identical subunits is expressed in a given reaction (Table I). Four mechanisms can be envisioned and there are shown schematically in Figure 5. The alternatives described are shown for a dimer where only one site is expressed. Alternative A suggests that the subunits are not identical, although their identity has been indicated by conventional tests. Such a change could result from a single amino acid replacement which alters the binding site significantly. Since isozymes are known and single amino acid substitutions are known, this mechanism is certainly plausible. A second possibility envisages identical polypeptide chains which dimerize in an asymmetric fashion so that the conformations of the two active sites are not identical. Not only is such an association theoretically possible, but it has now been observed in the case of insulin as subunits come together in an asymmetric manner in the insulin crystal (Adams *et al.*, 1969). A third possibility is that in which the active sites are sufficiently close together that the binding of one molecule sterically blocks or electrostatically repels the second molecule. Finally, a ligand-induced model can be considered in which it is assumed that the two subunits are identical and the dimer contains two identical active sites in the absence of ligand. Upon binding of ligand, a conformational change is induced and transmitted across the subunit contacts so that the second site is turned off. This is a type of negative cooperativity which has been applied to the alkaline phosphatase dimer by Trentham and Gutfreund (1968), Simpson and Vallee (1970), and Detitdems *et al.* (1970), and also for malic dehydrogenase by Harada and Wolfe (1968).

In the case of CTP-synthetase the evidence presented here indicates that the subunits have identical primary structures. No differences were observed on electrophoresis under conditions in which slight differences in other proteins have been shown to give multiple bands.

The second possibility is that the subunits are initially identical but become quasiequivalent upon dimerization producing an asymmetric dimer. This probability seems unlikely in the case of CTP-synthetase since Michaelis–Menten binding and a Hill coefficient of 1.0 are observed for GTP in the dimer and for ATP in the presence of UTP, and for UTP in the presence of ATP. Furthermore, a hyperbolic saturation curve for glutamine is observed in the tetramer in the absence of GTP or in the presence of saturating GTP. This would, at the minimum, suggest that the GTP sites in the dimer and the sites for ATP, UTP, and glutamine in the tetramer are identical. Thus, it seems that *a priori* all sites, including the glutamine sites, are equivalent and the possibility of asymmetric assembly appears unlikely. Furthermore, the insulin analogy must be viewed with caution since the asymmetry in that case is only at the point of subunit contacts and does not extend throughout the molecule. Moreover, this asymmetry has only been observed in the crystal and has not been shown in the dimers in solution. It could, therefore, be a property of compression during crystallization.

The third possibility that the sites are contiguous and steric hindrance or electrostatic repulsion prevents binding of the second molecule also seems unlikely. The possibility of the two DON molecules being so close together that they interfere with each other whereas the two molecules of glutamine are sufficiently far apart that they can bind without steric obstruction is unlikely. So far, no set of sites has been shown by crystallography to be juxtaposed in the manner suggested here. In fact, all tetrameric or oligomeric protein appear to have completely distinct regions in which the active sites are removed from the subunit contact regions. This is certainly the case in hemoglobin and lactic dehydrogenase and might be a general feature of protein design. The CTP-synthetase reaction is not enormously sensitive to changes in ionic strength and the substrate glutamine has the same charge effects as the DON reactant. Therefore, electrostatic repulsions seem implausible also.

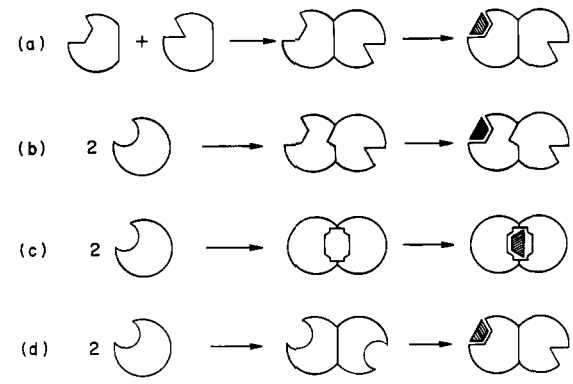

FIGURE 5: Possible sources for the half-of-the-site reactivity. (a) Nonidentical subunits with different sites. (b) Subunits are identical, but upon an asymmetric association to form a dimer one of the sites is deformed. (c) Two sites are adjacent and the first substrate molecule sterically blocks the second molecule. (d) The binding of one ligand induces a conformational change which alters the vacant site so that it cannot bind substrate.

The final alternative is a ligand-induced conformational change which renders the second site incapable of reaction after the first site has reacted. The reactivity of the sulfhydryl groups shows that the binding of DON induces a major conformational change (Figure 5). The conformation of the DON-enzyme is indeed different from that of the enzyme in the presence of saturating glutamine, as judged by the reactivity toward DTNB (Figure 4). Also, the DON-enzyme has a significantly different isoelectric pH from the native enzyme. Since the binding of DON does not add a net charge to the enzyme, the different electrophoretic behavior is probably due to a change in conformation. The binding of DON to the tetramer has a biphasic character (Figure 1, Table I), indicating that the ligand-induced conformational change extends not only to the neighboring subunit in one dimer pair, but extends across the other subunit contacts to affect the second dimer of the tetramer. Furthermore, this induced effect between dimer pairs can be abolished by addition of the allosteric effector, GTP, which is in agreement with the assumption that allosteric effectors can alter subunit interaction constants. This is a strong indication that DON acts *via* ligand-induced changes. The fact that glutamine can bind to the second site of the dimer but DON abolishes this binding in the alternate site again is best explained by a ligand-induced change.

If indeed the mechanism is correct, why is there such a dramatic difference between glutamine and DON, which are

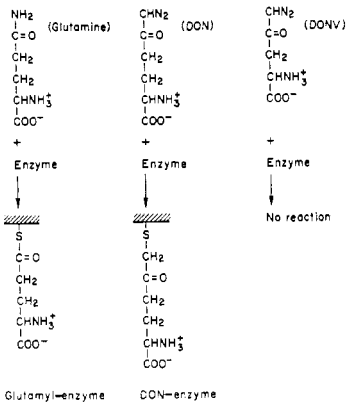

FIGURE 6: The interaction of CTP-synthetase with glutamine and DON. The structure of DON is compared to that of glutamine. A schematic presentation of the glutamine binding as compared to the DON binding is given.

structurally very similar? It has become apparent recently that small changes in a molecule can have dramatic effects on the rest of the molecule. For example, Perutz in his brilliant studies on hemoglobin has shown that the binding of an oxygen molecule to hemoglobin induces profound conformational changes because of the induced movement by 0.8 Å of the Fe^{2+} atom (Perutz, 1970). The presence of an extra carbon atom in DON compared to glutamine could be sufficient to trigger the same type of changes (Figure 6).

A more plausible explanation, however, is that the major conformational change induced by glutamine occurs when the covalent-glutamyl-enzyme intermediate is formed. This glutamyl-enzyme is constantly hydrolyzing and therefore only a small fraction of the subunits are at any one time in the form of the glutamyl intermediate (Levitzki and Koshland, 1970, 1971). On the other hand, DON forms an irreversible covalent linkage thus inducing the conformation change in that subunit and permanently changing the dimer to which it is attached.

The above arguments lead to the conclusion that the ligand-induced changes are the most plausible explanation for half-of-the-sites reactivity of CTP-synthetase, and a glance at Table I indicates that this could be the general mechanism for such reactivity. Insufficient evidence is at hand to establish a unique mechanism for each of these cases, but the ligand-induced mechanism can easily accommodate all the known cases. In several of the enzymes, listed in Table I, such as alkaline phosphatase, the polypeptide chains are known to be a product of a single gene and therefore amino acid differences in the primary structure cannot explain the half-of-the-sites reactivity. In the case of alkaline phosphatase, the half-of-the-sites reactivity in the dimer can be relieved either by changes in solution conditions or by chemical modification of the enzyme (Trentham and Gutfreund, 1968; Detitdems *et al.*, 1970; Simpson and Vallee, 1970). Furthermore, preliminary crystallographic studies on alkaline phosphatase (Hanson *et al.*, 1970) indicate that the two subunits have identical conformations in the absence of ligand.

Type of Conformation Changes Induced by DON. The reactivity of CTP-synthetase toward the affinity-label DON allows one to deduce a number of features of the subunit interactions which are illustrated in Figure 7.

In the first place, the dissociation of the tetramer to dimers and the lack of monomers (in the absence of denaturing conditions) indicates there are different types of subunit contacts in different directions for an individual subunit, *i.e.*, isologous

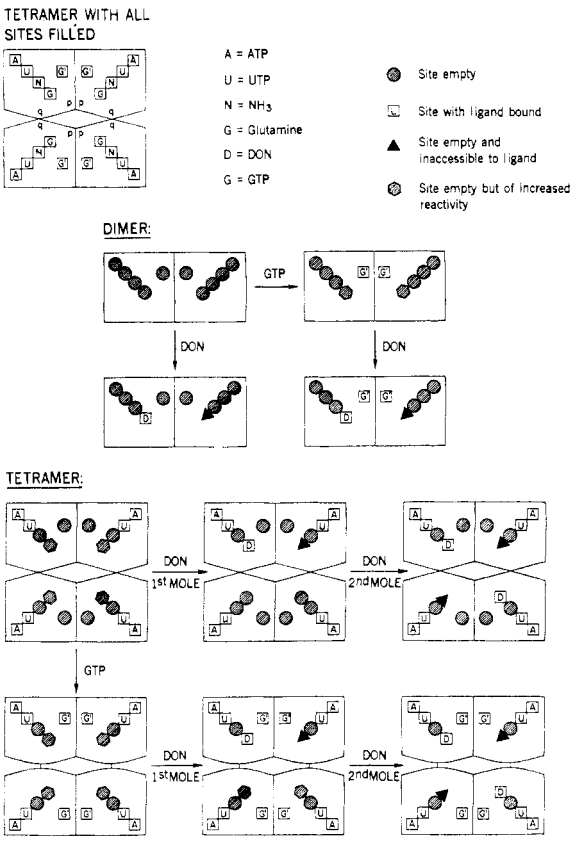

FIGURE 7: The reactivity of CTP-synthetase toward DON. Dimer: GTP increases the reactivity of the glutamine site toward DON tenfold. Both in the presence and absence of GTP only one out of two sites is labeled. Tetramer: the reactivity of the first site toward DON is tenfold higher than the second site. The reactivity of the first site on the tetramer is similar to the reactivity of the site on the dimer in the presence of GTP. The reactivity of the second site is similar to the dimer reactivity in the absence of GTP. The two sites on the tetramer react independently with DON in the presence of GTP.

binding is required as shown by the pp and qq domains, in Figure 7. The major and strongest effect of DON reaction is transmitted along the pp domain so that the alternate site within this dimer is then changed so much that subsequent reaction with either DON or glutamine is prevented. Whether the change is transmitted by a new shape which does not react or by a conformation which is frozen so that a necessary ligand-induced change to obtain reaction is prevented, cannot be ascertained directly. However, this altered reactivity of the unfilled glutamine site is indicated by a change from a hexagon to a filled triangle. The reaction of the first DON in the tetramer induces a conformation change which also extends across the qq domains and affects the sites in the second dimer. This is indicated by the changed reactivity toward the second mole of DON (Figure 1).

The molecule in Figure 7 is shown for convenience as a planar arrangement with each subunit in contact with two other subunits. The arrangements apply equally well to a tetrahedral arrangement with each subunit in contact with three other subunits (Cornish-Bowden and Koshland, 1970). This is illustrated in Figure 8. Although all of the subunits are initially identical, the binding of the first molecule of DON alters the conformations to require the order of binding of the subsequent molecule. If the pp domain is the main contact in the dimer, the first DON will alter the conformation of this

adjacent subunit so that it cannot react. A listing of the subunit interactions in b and c now show that the remaining two subunits do not have identical interactions and therefore one of the two final arrangements will be preferred on kinetic or thermodynamic grounds.

The reactivity of DON is also affected by binding of other ligands to the protein molecule. GTP not only affects the rate of reaction in the dimer but also influences the interactions across the qq plane in the tetramer.

Oligomeric Enzymes as Multiple of Dimers. It is interesting to note that all of the enzymes listed in Table I which are not dimers are capable of dissociating to dimers. It is attractive then to propose that all the hexamers and tetramers listed in Table I are composed of isologous dimers (Monod *et al.*, 1965). No case where a tetrameric enzyme is believed to be all heterologous was found to exhibit the property of half-of-the-sites reactivity. It would seem, therefore, that enzymes composed of isologous dimers can exhibit strong negatively cooperative intradimer subunit interactions which can bring about the chemical expression of only half of the sites. A way of visualizing this is the focusing of certain conformational changes across the pp binding domain. This would cause only half of the sites to react. A lesser but, nevertheless, significant effect across the qq domains would lead to reaction at every alternate subunit.

Independence of Sites. A striking feature of this study is the finding that profound conformational changes induced by DON can leave certain other activities of the protein unchanged. The chemistry of the CTP-synthetase reaction (Levitzki and Koshland, 1971) indicates a close geometric alignment of ATP, UTP, and glutamine. Moreover, it appears quite certain that the glutamylation of the enzyme releases ammonia at an ammonia site which is common both to the glutamine and ammonia reactions. When the enzyme is saturated with ATP and UTP, the ammonia site is unchanged upon binding of DON (Table III). Moreover, the Michaelis–Menten parameters show ATP and UTP reaction appears largely unaffected by DON modification at saturation (Table III). The primary effects of the conformation changes induced by DON, therefore, are those transmitted to glutamine sites of neighboring subunits with little, if any, effect on the subsites for ATP, UTP, and ammonia in the same active site. This is indicated schematically in Figure 7 by leaving the ATP, UTP, and NH_3 subsites unchanged in conformation as a result of the DON reaction.

Number of Conformation States. This study provides evidence of a large number of functional conformational states of the protein CTP-synthetase. Not only are there hybrid conformational states in which there is a progressive deformation of subunits as a single type of ligand binds, but also the conformational changes induced by one ligand may affect some subsites little and other sites greatly. This wide complexity of conformational states will depress some, since it would appear that this adds further complications to an already complex subject. In some ways it does, since it certainly diminishes the chances that some gross parameter such as optical rotatory dispersion will be able to make a nice correlation with the shape of the subunit as a whole. On the other hand, it does furnish a great added simplicity. The principle of flexibility, which has been shown to pervade not only specificity but also allosteric regulation, repressors, membranes, transport, and receptors is seen to operate as a simple general mechanism for the communication of information through protein molecules. Furthermore, it appears that nature has been sufficiently clever so that classes of functions can also be

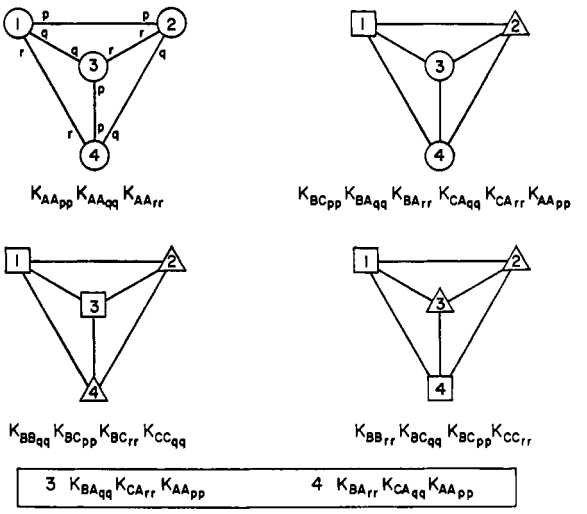

FIGURE 8: Induced subunit interactions in a tetramer which can explain DON-type reactivity. All isologous tetramer (in upper left hand corner) has all subunits with identical interactions. Binding of first DON changes subunit 1 to new conformation (square) and induces change in subunit connected by pp binding domain (triangle). This protein (upper right-hand corner) does not have identical three and four subunits because of differing binding domains connecting them to subunits 1 and 2. This is illustrated in portion of figure where second molecule of DON is bound, inducing change in subunit attached through pp domain. The two alternatives have differing subunit interactions and therefore one may be, and probably will be, appreciably more stable than the other.

separated within a protein molecule by proper protein design. Negative cooperativity with respect to one ligand need not imply negative cooperativity with respect to all ligands. In fact, CTP-synthetase shows strong positive cooperativity toward the binding of ATP and UTP (Long and Pardee, 1967) but negative cooperativity for DON and GTP (Long *et al.*, 1970; Levitzki and Koshland, 1969). This means that the same protein can be extraordinarily sensitive to control for two of its substrates and be desensitized toward the environmental fluctuations of a third substrate or an allosteric effector. Biological molecules are obviously designed to carry out more than one function and it should not be surprising that the communicating system within the protein is designed to allow some independence in the transmission of these signals, not unlike the signalling in a big city telephone system.

References

Adams, M. J., Blundell, T. L., Dodson, E. J., Dodson, G. G., Vijayan, M., Baker, E. N., Harding, M. M., Hodgkin, D. C., Rimmer, B., and Sheat, S. (1969), *Nature (London) 224*, 491.

Bernhard, S. A., Dunn, M. F., Luisi, P. L., and Schack, P. (1970), *Biochemistry 9*, 185.

Cornish-Bowden, A., and Koshland, D. E., Jr. (1970), *J. Biol. Chem. 245*, 6241.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Detitdems, C., Lazdunski, C., Chappelet, D., Molchin, A., and Lazdunski, M. (1970), *Eur. J. Biochem. 14*, 30.

Ellman, G. (1959), *Arch. Biochem. Biophys. 82*, 70.

Givol, D. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 5*, 153.

Hanson, A. W., Applebury, M. L., Coleman, J. E., and Wyckoff, M. W. (1970), *J. Biol. Chem. 245*, 4975.

Harada, K., and Wolfe, R. G. (1968), *J. Biol. Chem. 243*, 4123, 4131.

Hartman, S. C. (1963), *J. Biol. Chem. 238*, 3036.
Hedrick, J. L., and Smith, A. J. (1968), *Arch. Biochem. Biophys. 126*, 155.
Janin, J., van Rapenbusch, R., Truffa-Bachi, P., and Cohen, G. N. (1969), *Eur. J. Biochem. 8*, 128.
Koshland, D. E., Jr. (1970), *in* The Enzymes, 3rd ed, Vol. 1, Boyer, P. D., Ed., New York, N. Y., Academic Press, p 342.
Levitzki, A., and Koshland, D. E., Jr. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 1121.
Levitzki, A., and Koshland, D. E., Jr. (1970), *Biochim. Biophys. Acta 206*, 473.
Levitzki, A., and Koshland, D. E., Jr. (1971), *Biochemistry 10*, 3365.
Long, C. W., Levitzki, A., and Koshland, D. E., Jr. (1970), *J. Biol. Chem. 245*, 80.
Long, C. W., and Pardee, A. B. (1967), *J. Biol. Chem. 242*, 4715.
Malhotra, O. P., and Bernhard, S. A. (1968), *J. Biol. Chem. 243*, 1243.
Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.
O'Leary, M., and Westheimer, F. H. (1968), *Biochemistry 7*, 913.
Perutz, M. F. (1970), *Nature (London) 228*, 726.
Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962), *Nature (London) 195*, 281.
Rowe, P. B., and Wyngaarden, J. B. (1968), *J. Biol. Chem. 243*, 6373.
Simpson, R. T., and Vallee, B. L. (1970), *Biochemistry 9*, 953.
Tate, S., and Meister, A. (1961), *Proc. Nat. Acad. Sci. U. S. 68*, 781.
Trentham, D., and Gutfreund, H. (1968), *Biochem. J. 106*, 455.
Williams, D. E., and Reisfeld, R. A. (1964), *Ann. N. Y. Acad. Sci. 121*, 373.
Wilson, D. B., and Hogness, D. S. (1964), *J. Biol. Chem. 239*, 2469.
Wilson, D. B., and Hogness, D. S. (1969), *J. Biol. Chem. 244*, 2132.
Wrigley, C. (1968), *LKB Instrum. J. 15*, 2.

Studies on the Roles of the Catalytic and Allosteric Sites in Modulating the Reactivity of Tryptophan Oxygenase with Heme Ligands. I. Cyanide Derivatives*

Katsuro Koike† and Philip Feigelson‡

ABSTRACT: Spectral studies on bacterial tryptophan oxygenase and its cyano complex were performed. Ferritryptophan oxygenase at pH 7.0 exhibits spectral and electron paramagnetic resonance properties indicative of a single ferriheme moiety in a high-spin state ($d^5_{5/2}$). The monocyano complex, however, exhibits spectral and electron paramagnetic resonance properties typical of ferriheme in a low-spin state ($d^5_{1/2}$). From equilibrium binding data, dissociation constants (K_D), Hill coefficients (n), and standard free-energy changes were determined for the binding of cyanide to ferritryptophan oxygenase in the presence and absence of tryptophan. Either the substrate, tryptophan, or the competitive inhibitor, 5-fluorotryptophan, enhanced the affinity of ferriheme for cyanide (*i.e.*, lowered K_D), while the allosteric effector, α-methyltryptophan, was incapable of affecting this binding equilibrium (*i.e.*, K_D unchanged). It has been shown that α-methyltryptophan stabilizes the quaternary structure of the native enzyme and converts the "S"-shaped substrate saturation curve into a hyperbolic one. Hence, the affinity of the heme for cyanide is insensitive to certain cooperative interactions among subunits which modulate the catalytic activity of the native tetrameric enzyme molecule but rather seems to depend solely on saturation of the catalytic site by the substrate (tryptophan) or the competitive inhibitor (5-fluorotryptophan).

Tryptophan oxygenase is a dioxygenase, catalyzing the reaction between tryptophan and oxygen, yielding formylkynurenine (Tanaka and Knox, 1959; Feigelson *et al.*, 1965; Ishimura *et al.*, 1967). The tryptophan oxygenase of *Pseudomonas acidovorans* is a tetrameric heme protein of molecular weight 122,000 (Poillon *et al.*, 1969). Recent studies indicate the heme prosthetic group may oscillate in valence during catalysis (Forman and Feigelson, 1971). Its prosthetic heme also reacts with suitable ligands to form both ferri and ferro derivatives with characteristic absorption and epr spectra. In a recent report (Ishimura *et al.*, 1967), a close similarity was noted of the ternary complex (tryptophan–oxygen–ferrotryptophan oxygenase) detected spectrally to those of oxymyoglobin and horseradish peroxidase (III).

Evidence has been accumulating indicating that tryptophan oxygenase from *P. acidovorans* possesses regulatory as well as catalytic site(s) (Feigelson and Maeno, 1967; Koike *et al.*, 1969). The substrate, tryptophan, is capable of binding to both the regulatory and catalytic site(s); whereas, under

* From The Institute of Cancer Research and the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York 10032. *Received March 3, 1971.* This research was supported in part by U. S. Public Health Service Grant CA-02332.

† Present address: Laboratory of Molecular Genetics, Osaka University, Osaka, Japan.

‡ Career Investigator of the Health Research Council of the City of New York (I-104).