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Thermodynamics of Binding to Native α-Chymotrypsin and to Forms of α-Chymotrypsin in Which Catalytically Essential Residues Are Modified; a Study of "Productive" and "Nonproductive" Associations†

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ABSTRACT: The standard free energy ($\Delta G°$), enthalpy ($\Delta H°$), and entropy ($\Delta S°$) of association for proflavin and D- and L-*N*-AcTrp have been obtained at pH 7.8 for native α-chymotrypsin (Cht) and for forms of Cht in which essential catalytic residues of the active site are modified. The modified Cht forms studied are dehydroalaninyl-195-α-Cht and *N*-methylhistidinyl-57-α-Cht. Associations to native Cht (pH 7.8) are characterized by negative $\Delta H°$ and $\Delta S°$ values (i.e., for L-AcTrp $\Delta H° = -9.1$ kcal/mol and $\Delta S° = -21$ eu at $T = 25$ °C). In contrast, we found associations to modified Chts to be characterized by an enthalpy near zero and a positive entropy of association, the values of the $\Delta H°$ and $\Delta S°$ for association to the modified Cht forms being similar to those expected for transfer of small aromatic molecules from water to a nonpolar solvent phase. Differences in $\Delta H°$ and $\Delta S°$ observed for binding of substrate analogues and inhibitors to modified and native Cht (pH 7.8) are approximately +10 kcal/mol and +30 eu, respectively. Data from D. D. F. Shiao ((1970), *Biochemistry 9,* 1083) similarly show differences of comparable magnitude between binding of substrate analogues to active α-Cht (pH 7.8) and the His-57 protonated form of α-Cht (pH 5.6). The negative $\Delta H°$ and $\Delta S°$ values of associations for binding to active α-Cht indicate that a substrate-induced conformational change occurs on substrate association with the primary binding site (S_1), which does not occur in Ser-195 and His-57 modified Cht. From these differences we infer a linkage between binding of substrate into S_1 and the catalytic residues in the nucleophilic subsite (S_1–S_1'). Our data also show that associations of substrate analogues into potentially productive Michaelis complexes S_1 cannot be easily differentiated from associations that are nonproductive (i.e., nonactivated) from their $\Delta G°_{obsd}$, but may be differentiated by their respective $\Delta H°_{obsd}$ and $\Delta S°_{obsd}$ for association. Accordingly, it is indicated that the probable substrate *association–activation* process, characterized thermodynamically in this work, occurs in the substrate binding step and leads to lowered free energies of activation in catalytic steps succeeding binding; however, the process does not influence the observed strength of substrate binding.

The concepts of induced fit, substrate distortion, and productive substrate orientation have received wide support as mechanisms that may make a significant contribution toward lowering reaction activation energies in enzyme-catalyzed reactions (Jencks, 1975). In most of these hypotheses, it is argued that a favorable free energy of productive substrate binding into the noncovalent Michaelis complex is partially utilized to lower the activation energy of succeeding covalent bond making or breaking steps in the enzymic mechanism. This is accomplished by either (i) a conformational change in the enzyme active site induced by substrate binding leading to a precise orientation of catalytic residues of the enzyme and the reactive bond in the substrate (Koshland and Neet, 1968), (ii) distortion of the reactive bonds of the substrate on binding toward the transition state for the enzyme-catalyzed reaction (Eyring et al., 1954; Jencks, 1969a), or (iii) simply by the restriction of translational and rotational degrees of freedom in the substrate on binding with respect to the catalytic residues of the enzyme active site (Storm and Koshland, 1970; Bruice, 1970; Page and Jencks, 1971). These mechanisms of substrate and/or enzyme activation toward catalysis occurring in the noncovalent Michaelis complex between enzyme and substrate will be referred to in the following discussion as association–activation mechanisms. More precisely, association–activation describes the activation of substrate and/or enzyme toward catalysis on association with an enzyme active site prior to any covalent bond making or breaking step in the enzymic mechanism. The term encompasses both orientation and substrate distortion hypotheses.

In the case of chymotrypsin there is evidence in support of all three types of association–activation mechanisms (types

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i–iii above). Extensive quantitative kinetic evidence in support of the importance of substrate orientation as a significant acceleration factor in the Cht[1] mechanism has come from the study of Cht specificity with substrates whose structure has been systemically modified (Cohen and Schultz, 1967, 1968; Cohen, 1969). Experimental support for a substrate distortion type of association–activation mechanism in Cht may be inferred from the x-ray structure of pancreatic trypsin inhibitor–trypsin complex which shows the "reactive" peptide bond of the inhibitor to be partially distorted toward the tetrahedral transition state for the acylation reaction (Rühlmann et al., 1973). In addition there are some data on Cht that indicate a change in enzyme conformation occurs on substrate binding into the Michaelis complexes prior to the acyl-enzyme intermediate formation, indicating an induced-fit type of mechanism in Cht (Fink, 1976; Hess et al., 1970; Yu and Viswantha, 1969; Cruickshank and Kaplan, 1975; Havsteen, 1967). However, with the distortion and induced-fit type of mechanisms, the possible quantitative catalytic contribution of these mechanisms to the enzymic rates has not been established.

In order to understand the mechanism by which an enzyme catalyzes a reaction, it is necessary to elucidate the thermodynamic basis for each of the catalytic steps in the enzyme reaction. However, the thermodynamic data on the free energy, enthalpy, and entropy for substrate association and catalysis by the Cht active site are not well understood (Lumry and Biltonen, 1969; Jencks, 1975).

In this paper we compare thermodynamic values for substrate analogue associations to active α-Cht with thermodynamic values for associations to forms of α-Cht in which essential catalytic residues have been modified. The modified forms studied are those in which the hydroxymethyl side chain of Ser-195 has been chemically modified to dehydroalanine, and the His-57 methylated on its N-3 imidazole atom. The Ser-195 and His-57 side chains form a part of the catalytically important charge relay system of the Cht nucleophilic site and participate in the transition state for the enzyme-catalyzed reaction (Bender and Kézdy, 1964; Blow, 1976). Accordingly, they should play a role in substrate association–activation mechanisms. Thermodynamic differences found between binding to native α-Cht and to forms of Cht in which catalytically essential residues are rendered inert may offer insight into the thermodynamic basis for association–activation processes in Cht.

Experimental Section

α-Cht (CDI, 3× crystallized) and lima bean trypsin inhibitor were obtained from Worthington Biochemical Co. Proflavin dihydrochloride dihydrate was obtained from Mann Chemical Co. and found to be homogeneous by thin-layer chromatography (TLC) in 4:1:1 acetic acid/butanol/water; we found an ϵ_{444} of 3.66×10^4 M^{-1} cm^{-1} at pH 7.8, in agreement with previously published values (Brandt et al., 1967; Glazer, 1965). *N-trans*-Cinnamoylimidazole, L- and D-*N*-acetyltryptophan, AcTyrOEt, and turkey ovomucoid trypsin inhibitor were obtained from Sigma Chemical Co. *p*-Toluenesulfonyl chloride was purchased from Eastman Chemical Co. PheMeSO$_2$F and methyl *p*-nitrobenzenesulfonate were obtained from Pierce Chemical Co.

The normalities of α-Cht solutions were determined by titration with *N-trans*-cinnamoylimidazole (Schonbaum et al., 1961). Activity measurements were made spectrophotometrically utilizing AcTyrOEt as substrate (Schwert and Takenaka, 1955). Protein concentration was determined at 280 nm utilizing an extinction coefficient of 5.0×10^4 M^{-1} cm^{-1} (Dixon and Neurath, 1957).

Dehydroalanine-195-α-Cht (anhydro-Cht, ACht) was prepared by modification of the procedure of Ako et al. (1974). To 400 mg of α-Cht in 40 mL of sodium phosphate buffer (0.05 M, pH 7.8) at 2 °C were added 2 250-μL aliquots of stock PhMeSO$_2$F in acetone (28 mg/mL) at 15-min intervals. After 30 min the sample showed 0.1% of its original activity toward AcTyrOEt. The solution was brought to pH 13 with cold 6 N NaOH for 1.25 h and brought back to pH 7.8 with 6 N HCl, a third 250-μL aliquot of PhMeSO$_2$F was added, and after 30 min the solution was brought to pH 5 with 6 N HCl, dialyzed against cold water at pH 5, and lyophilized. The ACht was then purified in 100-mg aliquots by affinity chromatography over a lima bean trypsin inhibitor–Sepharose resin as described (Ako et al., 1974), except the pH 2 eluent from the column was brought to pH 5 prior to dialysis and the sample was dialyzed against pH 5 water. We found that when dialyses were carried out at pH 5, only minimal amounts of insoluble protein were present in the product after lyophilization (Ako et al., 1974), thus increasing the yield of usable ACht significantly.

N-Methylhistidinyl-57-α-Cht (MCht) was prepared according to the procedure of Ryan and Feeney (1975), except all dialyses were carried out at pH 5. As in the preparation of ACht, the pH 5 dialysis resulted in a completely soluble product. MCht samples had less than 1% of their original AcTyrOEt activity.

Binding constants for proflavin to native Cht, ACht, and MCht were calculated from difference absorption ($\Delta A_{obsd(465)}$) between proflavin and proflavin plus enzyme at 465 nm (Glazer, 1965; Bernhard et al., 1966; Brandt et al., 1967). The binding constants for L- and D-*N*-AcTrp to native Cht, ACht, and MCht were obtained from the decrease in $\Delta A_{obsd(465)}$ due to the competitive displacement of proflavin from the enzyme active site in solutions of known concentrations of proflavin, enzyme, and *N*-AcTrp (Glazer, 1965; Brandt et al., 1967). A T_m analyzer (Beckman Model 139000-W) modified for placement in the cell holder of a Cary-15 spectrophotometer was utilized to facilitate the collection of $\Delta A_{obsd(465)}$ data between 5 and 35 °C. The T_m analyzer consists of an electrically heated water-cooled cuvette chamber, an automatic temperature programmer, and a temperature bridge connected to a platinum resistance probe which measures the temperature of the sample solution in the cuvette. Temperature vs. ΔA_{obsd} readings are plotted on an x–y recorder with the x axis calibrated to read temperature directly from the platinum probe and the y axis light absorption units. At temperatures below 10 °C, the cuvette chamber was flushed with dry N_2 to prevent water condensation. The rate of heating was 1 °C/2 min. No differences in the $\Delta A_{obsd(465)}$ temperature plot were observed when the rate of heating was decreased by

[1] Abbreviations used are: Cht, α-chymotrypsin; ACht, dehydroalaninyl-195-α-chymotrypsin (anhydrochymotrypsin); MCht, $N^{\epsilon 2}$-methylhistidinyl-57-α-chymotrypsin; AcTyrOEt, *N*-acetyl-L-tyrosine ethyl ester; PhMeSO$_2$F, phenylmethanesulfonyl fluoride. S_i refers to the amino acid binding site in the active site for amino acid residue P_i in the substrate. Based on this nomenclature, a hexapeptide substrate $P_3 \cdots P_3'$ would productively align with sites $S_3 \cdots S'_3$ of the enzyme active site with the substrates' hydrolyzable bond between S_1–S'_1 (Schechter and Berger, 1967). In eq 4 and 5, P_1 and P_2 refer to the first and second product of the enzymic reaction, in accordance with standard Cht nomenclature. In turn, the S_1 site can be divided into four subsites complementary to the four groups bound to the α carbon of L-AcPheOMe when bound productively to the S_1 site (see Figure 3). These are the aromatic binding site, ar; the acylamide binding site, am; the hydrogen site, h; and the nucleophilic site, n (Cohen, 1969). These subsites are more explicitly defined in the schematic diagram of Figure 3.

TABLE I: Thermodynamic Data for the Binding of Substrate Analogues to α-Cht.[a,b]

Substrate	T (°C)	$K_I \times 10^3$ (M)	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (eu)
Proflavin[c,e]	5.0	0.0106 ± 0.0013			
	25.3	0.0270 ± 0.0003	−6.23 ± 0.01	−8.01 ± 1.09	−6 ± 4
	35.0	0.0443 ± 0.0031			
L-AcTrp[d,e]	5.0	2.69 ± 0.30			
	25.3	6.97 ± 0.73	−2.94 ± 0.07	−9.06 ± 0.96	−21 ± 3
	35.0	13.9 ± 0.7			
D-AcTrp[d,e]	5.0	1.31 ± 0.20			
	25.3	4.27 ± 0.68	−3.23 ± 0.10	−10.0 ± 1.3	−23 ± 4.5
	35.0	7.80 ± 0.58			

[a] T = 25.3 °C. [b] At pH 7.8, 0.05 M in sodium phosphate, 0.1 M NaCl. [c] [Proflavin] = 3.5×10^{-5} M; [Cht] = 2×10^{-5} M; $\Delta\epsilon_{465} = 2.45 \times 10^4$ M^{-1} (5 °C), 2.23×10^4 M^{-1} (25 °C), 2.18×10^4 M^{-1} (35 °C), K_I and $\Delta\epsilon_{465}$ being determined by titration with enzyme (Glazer, 1965). [d] [Proflavin] = 3.5×10^{-5} M, [Cht] = 2×10^{-5} M, [AcTrp] = 1×10^{-2} M; K_I determined according to Brandt et al. (1967), with K_{SP} and $\Delta\epsilon_{465}$ for proflavin–AcTrp of 0.139 M and 5.83×10^3 M^{-1} at 25 and 35 °C, and K_{SP} and $\Delta\epsilon_{465}$ for AcTrp–proflavin of 0.106 M and 5.54×10^3 M^{-1} at 5 °C. [e] Calculation of thermodynamic values by method 1 (see Experimental Section).

a factor of 2 or more, showing the solution was reaching quasi-temperature equilibriums under the conditions of our experiment. After a plot was obtained between 5 and 35 °C, a sample could be recooled to 5 °C and the plot obtained a second time on the same sample with no significant change in the plot. This shows that autolysis of the enzyme and photodecomposition of proflavin are not significant on the time scale of our experiments. The use of the T_m analyzer minimizes pipetting errors in the comparison of binding constants from ΔA_{obsd} values at different temperatures. In experiments with D- or L-AcTrp, the sample cell buffer solution contained the appropriate concentration of AcTrp and the NaCl concentration in the buffer was decreased appropriately to preserve an equivalent ionic strength in all experiments. In some cases $\Delta A_{obsd(465)}$ values were checked at a particular temperature in a single experiment without use of the T_m analyzer. In these cases temperature was recorded directly with a Digitec Thermistor (United Systems Corporation, Dayton, Ohio). When the ΔA_{obsd} was low, a tandem mixing cell was utilized according to the procedures for difference spectroscopy described by Yankeelov (1963). Based on $\Delta A_{obsd(465)}$ values obtained for native α-Cht at pH 7.8, the equilibrium dissociation constants (K_I) and difference extinction coefficients ($\Delta\epsilon_{465}$) at 5.0, 25.0, and 35.0 °C were calculated for binding of proflavin to native α-Cht by the method of Glazer (1965). We found a K_I of 2.69×10^{-5} M and a $\Delta\epsilon_{465}$ of 2.23×10^4 M^{-1} cm^{-1} at 25 °C and pH 7.8, in agreement with values previously reported under these conditions (Brandt et al., 1967; Glazer, 1965). K_I values for L- and D-AcTrp from $\Delta A_{obs(465)}$ data were calculated according to the procedures previously described by Brandt et al., with appropriate correction for the absorption due to *N*-AcTrp and proflavin as described (Brandt et al., 1967). We found K_I values at 25 °C and pH 7.8 of 3.57 $\times 10^{-3}$ and 6.97×10^{-3} M for D- and L-*N*-AcTrp, respectively, in good agreement with the binding constants obtained from steady-state kinetic techniques for these compounds (Foster and Niemann, 1955; Cunningham and Brown, 1956). Values of K_I for the binding of proflavin and D- and L-*N*-AcTrp to MCht and ACht were similarly calculated according to the procedures described by Brandt et al. (1967). We obtained values of K_I and $\Delta\epsilon_{465}$ for the binding of proflavin and D- and L-*N*-AcTrp to MCht at 25 °C and pH 7.8 similar to those found for the binding to native α-Cht at 25 °C, in agreement with the binding constants of proflavin and substrates to MCht reported previously (Henderson, 1971). The binding constants obtained for L-AcTrp to ACht are similar to those obtained with native. This is in general agreement with the values reported previously for substrate analogue binding to S_1 in ACht (Weiner et al., 1966). The experimental conditions utilized to obtain K_I values to native Cht, MCht, and ACht are summarized in the tables.

The free energy ($\Delta G°$), enthalpy ($\Delta H°$), and entropy ($\Delta S°$) for association of substrate analogues to the active site of native and modified Cht were calculated from the temperature dependence of the dissociation constants by standard formulas (Dixon and Webb, 1964; Lumry and Rajender, 1971) utilizing one of two methods for treatment of data. In *method 1*, the mean binding dissociation constants found at 5, 25, and 35 °C were utilized to calculate the $\Delta G°$, $\Delta H°$, and $\Delta S°$ values. The error in the enthalpy and entropy of association was computed by the following formulas (eq 1 and 2) (Wiberg, 1964):

$$\delta = R\frac{T'T}{T' - T}\ln\frac{1+\alpha}{1-\alpha} \quad (1)$$

$$\sigma = \delta\left(\frac{1}{T}\right) + R\ln(1+\alpha) \quad (2)$$

where α is the fractional average standard deviation in K_I at 5 and 35 °C, δ is the error in the enthalpy, σ is the error in the entropy, and T and T' are the temperature extremes of the experiment. Based on this formula, an error of approximately ±10% in our K_I values will lead to an error of ±1.1 kcal/mol in the enthalpy and ±4.0 eu in the entropy. A maximal error of ±15% in K_I would lead to an error of ±1.7 kcal/mol in enthalpy and ±6.0 eu in entropy. In *method 2*, the $\Delta G°$, $\Delta H°$, and $\Delta S°$ values are computed for each experimental run in which the T_m analyzer was utilized. The reported thermodynamic values and error then represent the mean and standard deviation of the respective single determinations of $\Delta H°$, $\Delta S°$, and $\Delta G°$ from multiple experimental runs. In the T_m analyzer the relative variation of K_I with temperature is obtained on a single solution, thus eliminating random errors in pipetting, weighing, and active-site titration that are reflected in the α values in the computation of $\Delta H°$ from mean K_I values by method 1.

Calculation of T_c (see eq 6 in the Discussion) from our data can be made by plotting $\Delta H°$ vs. $\Delta S°$ for each substrate analogue or by dividing the $\Delta\Delta H°$ by the $\Delta\Delta S°$ for each substrate in Table IV.

TABLE II: Thermodynamic Data for the Binding of Substrate Analogues to Anhydro-Cht.[a,b]

Substrate	T (°C)	$K_I \times 10^3$ (M)	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (eu)
Proflavin[c]	5.0	0.63 ± 0.08			
	25.0	0.63 ± 0.07	−4.36 ± 0.07	−0.38 ± 1.45	+13 ± 5
	35.0	0.68 ± 0.08			
L-AcTrp[d]	5.0	10.0 ± 0.95			
	25.0	7.59 ± 1.24	−2.98 ± 0.24	+1.29 ± 0.79	+14 ± 3
	35.0	8.62 ± 1.74			
D-AcTrp[d]	5.0	30.4 ± 4.6			
	25.0	27.3 ± 5.5	−2.14 ± 0.12	+0.58 ± 0.39	+9.1 ± 1.7
	35.0	27.8 ± 5.8			

[a] T = 25.0 °C. [b] At pH 7.8, 0.05 M in sodium phosphate, 0.1 M in NaCl; K_I and $\Delta\epsilon_{465}$ values obtained by procedure of Brandt et al. (1967). [c] [Proflavin] = 3.5×10^{-5} M, [ACht] = 1.3 to 7.2×10^{-4} M; error in K_I calculated from standard error of intercept in weighted least-squares linear regression; thermodynamic parameters calculated by method I (see Experimental Section); $\Delta\epsilon_{465} = 1.77 \times 10^4$ (5 °C), 1.46×10^4 (25 °C), 1.33×10^4 (35 °C). [d] [L-AcTrp] = 1 to 2×10^{-2} M, [D-AcTrp] = 5×10^{-2} M, [ACht] = 1.2×10^{-4} M, [proflavin] = 3.5×10^{-5} M; $\Delta\epsilon_{465}$ and K_{SP} for AcTrp-proflavin same as in Table I; thermodynamic parameters calculated by method 2 (see Experimental Section).

TABLE III: Thermodynamic Data for the Binding of Substrate Analogues to Methylhistidinyl-57-Cht.[a,b]

Substrate	T (°C)	$K_I \times 10^3$ (M)	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (eu)
Proflavin[c]	5.0	0.0207 ± 0.0014			
	25.0	0.0257 ± 0.0011	−6.24 ± 0.04	−2.99 ± 0.70	+11 ± 2.5
	35.0	0.0372 ± 0.0021			
L-AcTrp[d]	5.0	21.5 ± 4.9			
	25.0	16.5 ± 2.4	−2.52 ± 0.16	+1.38 ± 1.36	+12 ± 4
	35.0	18.3 ± 1.1			
D-AcTrp[d]	5.0	5.51 ± 0.67			
	25.0	7.30 ± 0.61	−2.92 ± 0.05	−2.66 ± 0.77	+0.9 ± 2.5
	35.0	8.88 ± 0.85			

[a] T = 25.0 °C. [b] At pH 7.8, 0.05 M sodium phosphate, 0.1 M NaCl; K_I and $\Delta\epsilon_{465}$ values obtained by procedure of Brandt et al. (1967). [c] [Proflavin] = 3.5×10^{-6} M, [MCht] = 3 to 16×10^{-5} M, $\Delta\epsilon_{465} = 2.54 \times 10^4$ M^{-1} (5 °C), 2.26×10^4 M (25 °C), 2.10×10^4 M^{-1} (35 °C); error in K_I calculated from standard error of intercept in least-squares linear regression; thermodynamic parameters calculated by method 1 (see Experimental Section). [d] [AcTrp] = 1×10^2 M, [MCht] = 2.6×10^{-5} M, [proflavin] = 3.5×10^{-5} M, $\Delta\epsilon_{465}$ and K_{SP} for AcTrp-proflavin same as in Table I; thermodynamic parameters calculated by method 2 (see Experimental Section).

Results

The binding equilibrium constants found for proflavin and D- and L-*N*-AcTrp to native Cht, MCht, and ACht at three temperatures and at pH 7.8 are given in Tables I–III. These constants were calculated from the absorbance at 465 nm due to the proflavin–enzyme complex as described in the Experimental Section, assuming that 1 equiv of proflavin reversibly binds at the enzyme active site. This assumption has been previously shown to be true for native α-Cht and MCht (Glazer, 1965; Bernhard et al., 1966; Antonov and Vorotyntseva, 1972). Figure 1 shows a linear Scatchard plot obtained from our data for proflavin and ACht at 25 °C, which supports the assumption of a single binding site in ACht for proflavin (Scatchard, 1949).

Van't Hoff lots of the temperature dependence of K_I led to the standard enthalpy of association, and the free energy and entropy of association were then calculated at 25 °C by standard procedures (Dixon and Webb, 1964; Lumry and Rajender, 1971). Figure 2 shows a Van't Hoff plot of the temperature dependence of log $\overline{K}_I$ vs. $\overline{T}$. A close interpretation of the standard deviation of the points at 5, 25, and 35 °C indicates the existence of 2 straight lines intersecting at 23 °C, showing the possible existence of high- and low-temperature conformations with slightly different enthalpies for proflavin association. Evidence for the existence of high- and low-temperature conformations for α-Cht, which are present in equimolar amounts near 23 °C, has been previously reported (Kim and Lumry, 1971; Lumry and Biltonen, 1969). However, only minimal differences in Cht turnover rate and specificity were found between the two forms (Lumry and Biltonen, 1969). In our calculations of $\Delta H°$, the small discontinuity near 23 °C has been ignored, and the $\Delta H°$ values reported represent the average $\Delta H°$ between 5 and 35 °C. This procedure is in accord with the procedures of other investigators who have ignored small heat capacity changes in comparing the enthalpies of association of substrate analogues of similar structures and over a similar temperature range to those binding molecules studied herein (Shiao, 1970; Lumry and Biltonen, 1969; Berezin et al., 1970; Kim and Lumry, 1971; Lumry and Rajender, 1971).

The values obtained for the standard enthalpy and entropy of association at 25 °C and pH 7.8 are in qualitative agreement with those obtained in other laboratories for the association of similar compounds to native α-Cht at this pH (Vaslow and Doherty, 1953; Canady and Laidler, 1958; Lumry and Biltonen, 1969; Lumry and Rajender, 1971; Shiao, 1970). For example, the value found in this work for the enthalpy of association of proflavin to α-Cht is within experimental error of the value reported by Shiao and Sturtevant (1969) by flow microcalorimetry at 25 °C and pH 7.8. Also, our value for the

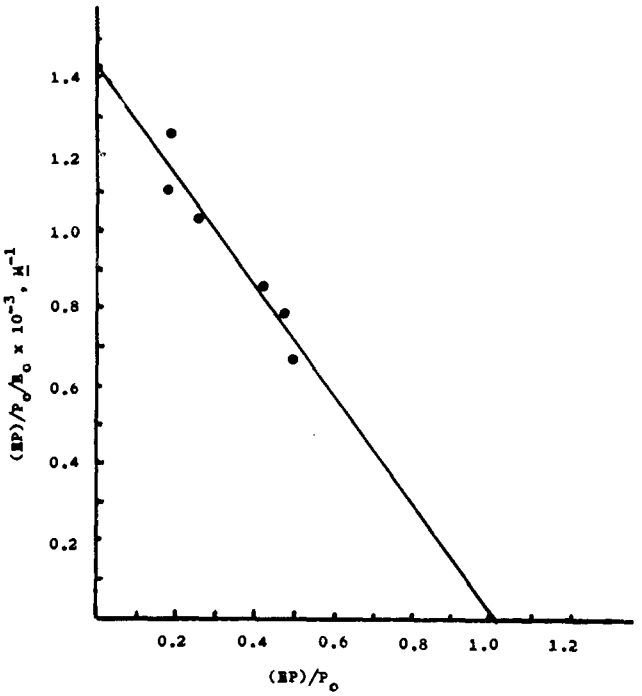

FIGURE 1: Scatchard type plot for binding of proflavin to anhydro-Cht.

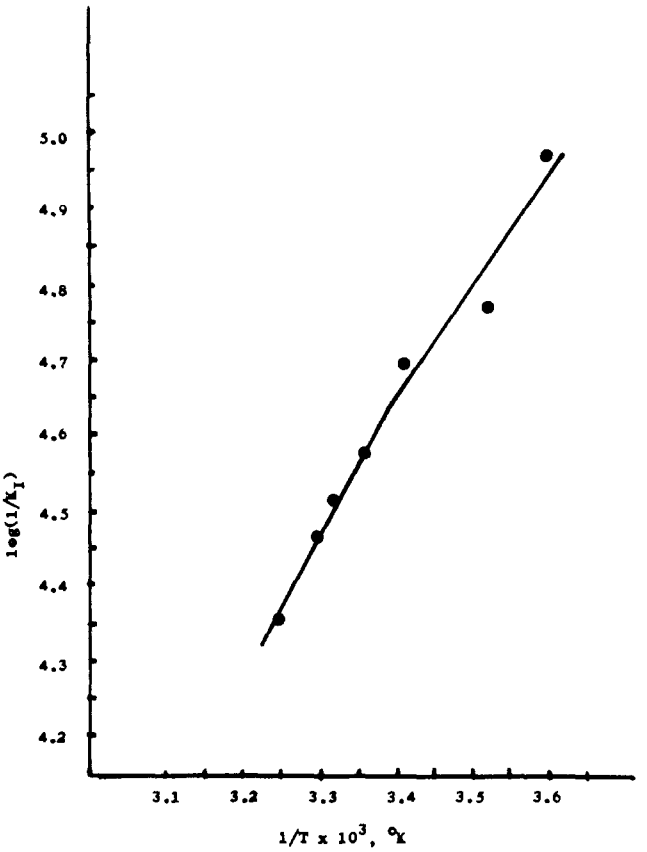

FIGURE 2: Van't Hoff plot of log $(1/K_I)$ vs. $1/T$ for binding of proflavin to native α-Cht at pH 7.8, in 0.1 M NaCl and 0.05 M sodium phosphate.

enthalpy of binding to native α-Cht of D-AcTrp is similar to that reported by Yapel (1967; see also Shiao, 1970), from the temperature dependence of binding between 3 and 13 °C at pH 8.0. However, our values for D-AcTrp are more positive than those reported by Shiao (1970). The enthalpy and entropy values we found for the association of L-AcTrp to native α-Cht are also more positive than in the reports of Yapel (1967) and Shiao (1970). Disagreement between various reports on the absolute enthalpy and entropy values for substrate analogue binding to α-Cht is not uncommon (Shiao, 1970). It may be that inherent in each of the techniques utilized for obtaining enthalpy of association there exist highly probable sources for consistent nonrandom error affecting the absolute values of the reported enthalpy of association. Shiao's data, for example, were obtained at high concentrations of enzyme (0.5×10^{-3} M) where most of the enzyme is present in polymer form (Shiao and Sturtevant, 1969; Pandit and Rao, 1975). Although corrections were made in his experiments for the heat of polymer dissociation to monomer on rapid dilution with solution containing substrate analogue in the flow microcalorimeter, such corrections may be more complex than realized due to disagreement over the possibility of substrate binding to the polymer form and the effect of substrate on the monomer-polymer equilibrium (Pandit and Rao, 1975; Nichol et al., 1972). Furthermore, it is well documented that the α covalent form of Cht can exist in a variety of pH, ionic strength, and temperature-dependent conformation forms at pH 7.8 and 25 °C (Lumry and Biltonen, 1969; Kim and Lumry, 1971; Roger et al., 1969; Corey et al., 1965; Fersht and Requena, 1971). In some cases equilibria between these forms are slow (Kim and Lumry, 1971; Corey et al., 1965; Fersht and Requena, 1971) and in other cases have not been characterized. The monomer unit within the polymer structure may have a different conformation than the free monomer (Horbett and Teller, 1974; Aune et al., 1971), and the initial monomer conformational form produced on dissociation of a monomer unit from the polymer aggregate may be conformationally different from the most thermodynamically stable monomer conformation. Such complications can lead to the differences in the thermodynamic values reported from Shiao and other laboratories. However, the differences in the absolute values in the enthalpy and entropy of association to native α-Cht found between different laboratories should not distract from general thermodynamic conclusions that can be made from these data. Binding of small aromatic substrate analogues to the S_1 site in native α-Cht between pH 7.0 and 8.5 is in all cases found to be characterized by a significantly negative enthalpy and entropy of association. This is in contrast to the enthalpy and entropy found for the transfer of a small aromatic molecule from water to a nonpolar phase, which is characterized by an enthalpy near zero and a positive entropy (Jencks, 1969b; Tanford, 1973).

In contrast to the temperature dependence of K_I found for binding to native α-Cht that led to a negative enthalpy of association (between −8 and −10 kcal/mol) and a negative entropy of association (between −6 and −23 eu) (Table I), our data clearly show that the free energy of binding to ACht is temperature independent leading to the enthalpy near zero and a positive entropy for association (Table II). The thermodynamic values for substrate analogue binding to MCht similarly show a significantly more positive enthalpy and entropy than found for binding to native α-Cht (Table III).

Discussion

Interpretations based on enthalpy and entropy data with complex molecules such as proteins are speculative as the observed thermodynamic values may represent a composite of complex processes including differences in substrate and/or enzyme solvation. However, the observed free-energy changes in substrate binding and catalysis are directly dependent on

TABLE IV: Differences in the Enthalpy and Entropy of Association between Modified Chymotrypsins and Active α-Chymotrypsin.

Modified Cht Form	Substrate or Inhibitor	$\Delta\Delta H^{\circ}$ [d] (kcal/mol)	$\Delta\Delta S^{\circ}$ [e] (kcal/mol)
ACht[a]	Proflavin	+7.6	+19
	L-AcTrp	+10.4	+35
	D-AcTrp	+10.6	+32
MCht[b]	Proflavin	+5.0	+17
	L-AcTrp	+10.4	+33
	D-AcTrp	+7.3	+24
Protonated Cht (pH 5.6)[c]	Hydrocinnamate	+9.9	+33
	Indole	+8.8	+30
	D-AcTrp	+14.0	+46

[a] Dehydroalaninyl-195-αCht. [b] *N*-Methylhistidinyl-57-α-Cht. [c] Native α-Cht at pH 5.6; data from Shiao (1970). [d] ΔH°_{assoc} to modified enzyme minus ΔH°_{assoc} to native enzyme (pH 7.8). [e] ΔS°_{assoc} to modified enzyme minus ΔS°_{assoc} to native enzyme (pH 7.8); entropy calculated for 25 °C.

the enthalpy and entropy changes for the various steps in the process (eq 3):

$$\Delta G = \Delta H - T\Delta S \text{ (second law of thermodynamics)} \quad (3)$$

Accordingly, any credible explanation for the mechanism of enzymic catalysis should be able to account for the observed enthalpy and entropy contributions to the observed free-energy changes of the various steps in the enzyme's mechanism (Lumry and Biltonen, 1969). While any current explanation of the enthalpy and entropy contributions to enzyme processes may be speculative, such explanations deserve careful attention in the effort to elucidate the energetic basis for enzyme catalysis.

Our results show significant differences between the enthalpy and entropy of association of substrate analogues and inhibitors to the S_1 site of active α-Cht at pH 7.8 and to forms of α-Cht in which catalytically essential residues in subsite n are modified (Tables I–III; Figure 3). The subtraction of the enthalpy and entropy values found for each substrate analogue or inhibitor for association to active α-Cht from that found for association to the modified Chts shows a change in enthalpy, $\Delta\Delta H^{\circ}$, and a change in entropy, $\Delta\Delta S^{\circ}$, of approximately +10 kcal/mol and +30 eu, respectively, between binding to active and modified Cht (Table IV). Included in Table IV are the differences in enthalpy and entropy found by Shiao between binding to α-Cht at pH 5.6 and 7.8 (Shiao, 1970). In our context, in the pH 5.6 form of Cht the His-57 is modified by protonation and, accordingly, it is a modified catalytically inert Cht form.

The more negative enthalpies and entropies of association found for binding to active α-Cht at pH 7.8 indicate that the binding of aromatic molecules into S_1 with association at ar (Hein and Niemann, 1961; Cohen, 1969) induces a conformational change in the enzyme that may be functionally significant. This interpretation based on the negative enthalpy and entropy found for binding of aromatic molecules to S_1 of active α-Cht has been made previously by others (Lumry and Biltonen, 1969; Lumry, 1974; Shiao, 1970). In contrast, the bindings of substrate analogues and aromatic molecules to ACht, MCht, and His-57 protonated Cht are characterized by more positive enthalpy and entropy values (Table IV), more similar to those found for the transfer of small hydrophobic molecules from water to a nonpolar organic solvent (Jencks, 1969b; Tanford, 1973). This indicates that on modification of the Ser-195 and His-57 residues in the n site, the proposed substrate-induced conformational change in the enzyme does not occur. Accordingly, these results support the existence of

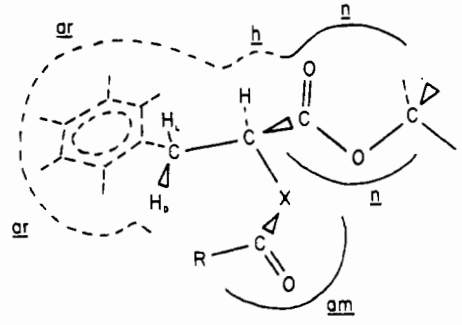

FIGURE 3: Schematic diagram of regions of the primary site (S_1) of Cht in productive association with L enantiomers of methyl α-substituted β-phenylpropionates; for substrate L-*N*-acetylphenylalanine methyl ester, X = NH, R = CH_3. Definitions of subsites of S_1 are: the aromatic binding site, ar, into which the β-aryl group of the substrate is held by hydrophobic interaction; the acylamide site, am, in which the L-α-amide NH of P_1 forms a hydrogen bond to the Ser-214 of Cht (Blow, 1976); the h site into which the α-D hydrogen is placed; and the nucleophilic site, n, into which the scissile bond of the substrate is placed and acted upon by the Ser-195 and His-57 within this site. These definitions are according to Cohen (1969).

a functionally significant linkage between binding to the S_1 site with association at ar and the catalytic residues of the n site.

It has been previously shown that the α-Cht-catalyzed hydrolysis of ester and amide bonds in substrates occurs by at least three distinct steps at pH 7.8:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+}{ES'} \xrightarrow[+H_2O]{k_3'} E + P_2 \quad (4)$$
$$P_1$$

where ES is the initial noncovalent Michaelis complex, ES′ is the covalent acyl-serine intermediate between the acyl portion of the substrate and the Ser-195 hydroxymethyl side chain, P_1 is the first product of the reaction (alcohol or amine), and P_2 is the acid product of the enzyme-catalyzed reaction (Bender and Kézdy, 1964). The linkage that is indicated from the thermodynamic data between substrate binding into S_1 and the Ser-195 and His-57 in n, within the noncovalent Michaelis complex, then suggests the addition of a second Michaelis intermediate on the pathway of substrate catalysis (ES_A):

$$E + S \underset{k_{-1a}}{\overset{k_{1a}}{\rightleftharpoons}} ES \underset{k_{-1b}}{\overset{k_{1b}}{\rightleftharpoons}} ES_A \xrightarrow{k_2} \underset{+}{ES'} \xrightarrow[+H_2O]{k_3'} E + P_2 \quad (5)$$
$$P_1$$

This is the activated intermediate in which substrate in association with the enzyme is activated toward catalysis. In this intermediate, the catalytically important Ser-195 and His-57 side chains of the n site are stabilized in an energetically activated configuration with respect to the bound substrate.

Experimental support for the existence of an additional Michaelis intermediate, ES_A, can be found in spectrophotometric, kinetic, and titration studies of α-Cht that have been previously reported. Fink has recently studied the α-Cht-catalyzed hydrolysis of L-AcPhe *p*-nitrophenylanilide at −90 °C in Me_2SO–water (Fink, 1976). At this temperature he spectrophotometrically observes three different Michaelis intermediates on the reaction pathway between ES and ES′ at pH 7.6. The last of these intermediates is not formed at pH <7 or on binding of the substrate to MCht (Fink, 1976). The characteristics for this third intermediate correlate with our postulated intermediate, ES_A, in that ES_A is also not apparent from the thermodynamic data at pH <7 or on substrate analogue binding to MCht. Fink estimates a rate constant at ambient temperatures of 1.4×10^3 s^{-1} for formation of this pH-dependent intermediate, which is at least an order of magnitude faster than the turnover rates found for Cht substrates (Fink, 1976). Other workers have also reported spectrophotometric observations of an intermediate between the initial ES complex and the acyl-enzyme (ES′) with chromophoric *N*-furylacryloyl substrates (Hess et al., 1970; Yu and Viswantha, 1969). In addition, Havsteen studied the binding of proflavin to α-Cht by temp-jump techniques and found the binding to be a two-step process at pH >7 (Havsteen, 1967). The rate constant for the isomerization step at pH >7 was found to be 525 s^{-1}, again on a time scale where this isomerization step can be mechanistically significant if it also occurs on substrate binding (Havsteen, 1967). Additional support for differences in the conformation of the catalytic residues of n site in the free enzyme and in the Michaelis complex comes from the observed perturbation of the pK_a of His-57 on substrate binding into ES (Fersht and Renard, 1974), and the differences observed in the chemical reactivity of the His-57 toward modifying reagent in free Cht and in Cht with indole bound in ar (Cruickshank and Kaplan, 1975).

The compensation observed in the enthalpy and entropy in the Cht process on perturbation of solution pH or substrate structure, resulting in minimal changes in the observed free-energy change for the process, has been previously discussed (Lumry and Biltonen, 1969; Lumry and Rajender, 1970; Lumry, 1974). If such enthalpy and entropy changes are plotted according to eq 6, it has been shown that linear plots may be obtained with values of T_c between 270 and 300 K for Cht processes (Lumry and Biltonen, 1969; Lumry and Rajender, 1970; Lumry, 1974). Slopes in this range of values are characteristic of reaction processes that occur in water, and it is inferred that the compensation is due to changes in water–solvent structure (Lumry and Biltonen, 1969; Lumry and Rajender, 1970; Lumry, 1974). Our data then extend the observation of such compensation phenomena between enthalpy and entropy with slope between 270 and 300 K to cases in which the structure of the Cht active site is chemically modified. The compensation appears real and not due to correlation of errors in enthalpy with entropy, based on the magnitude of the changes in $\Delta\Delta H°$ and $\Delta\Delta S°$ and the consistency of these changes. Furthermore, when entropy was computed from plots of $T \ln (\overline{K})$ vs. T (Lumry and Rajender, 1971), the values found are in close agreement with the values reported in Tables I–III.

$$\Delta H° = T_c \Delta S° + \text{constant} \qquad (6)$$

If the observed differences in thermodynamic parameters between binding to active α-Cht and modified α-Chts are due to solvation differences, as indicated by the T_c values, then we must conclude that either the free enzymes and/or the enzyme–inhibitor (substrate) complexes are solvated differently. As the modifications in Table IV only involve the addition or subtraction of small chemical grouping and the apparent effects on the thermodynamic parameters toward a more positive enthalpy and entropy are similar in all three modified enzyme cases (Table IV), we feel the data support differences in the enzyme–inhibitor (substrate) complex conformation between the modified enzymes and the active enzyme due to the prevention of association–activation in the modified enzymes. Low and Somero (1975) have recently presented data showing volume of activation changes related to transition-state formation in enzyme reactions. These protein volume changes correlated with formation of the transition state may be predicted to perturb the solvation shell of the protein and produce compensating thermodynamic changes in the enthalpy and entropy such as we have observed. We are suggesting that the conformational change inferred from the thermodynamic data on binding to active Cht is functionally significant, vide supra.

A conclusion that may be inferred from the thermodynamic data is that association–activation is not directly observable in the observed free energy of binding, but only in the enthalpy and entropy of binding to Cht. This conclusion is surprising, as supporters of association–activation hypotheses have previously argued that a part of the favorable free energy of binding in enzyme–substrate association is utilized to activate the substrate toward the transition state of the reaction catalyzed by the enzyme:

$$\Delta G°_{obsd} = \Delta G°_i + \Delta G°_a \qquad (7)$$

with

$$\Delta G°_i = \Delta H°_i - T\Delta S°_i, \qquad \Delta G°_a = \Delta H°_a - T\Delta S°_a$$

and where ΔG_i is the intrinsic (true) binding free energy and $\Delta G°_a$ is the energy utilized for substrate–enzyme activation. Accordingly, productive associations are expected to have a poorer observed free energy for binding (ΔG_{obsd}) than nonproductive associations of enzyme and substrates (Jencks, 1975). However, the observed free energies of substrate association to chemically modified inert forms of Cht are in the opposite direction from that predicted. In our example the catalytically essential Ser-195 and His-57 must participate in the transition state to which the substrate–enzyme complex is activated toward in association–activation, and therefore should play a role in association–activation processes. The prevention of productive associations by modification of these catalytically essential residues to the transition state of the enzymic reaction then eliminates probable pathways for the transfer of binding energy into substrate activation within the Michaelis complex. Accordingly, the observed binding free energy to modified Chts should be better than to the native enzyme, as $\Delta G°_a$ is zero (eq 7). However, *a better affinity to modified Chts is not observed* in this work with the virtual substrate L-AcTrp or in data from other laboratories in which the binding affinities of substrates or substrate analogues to modified Chts have been studied (Weiner et al., 1966; Henderson, 1971). A clearer understanding of the thermodynamics of association–activation may then come from consideration of the observed enthalpy and entropy contributions to $\Delta G°_{obsd}$ in Cht. Assuming that the initial driving force for association of substrate to the enzyme active site is the hydrophobic in-

teraction formed between the substrate side chain and the ar subsite in S_1 of the enzyme (Cohen, 1969; Hein and Niemann, 1961), one may expect from model thermodynamic studies of the transfer of small hydrophobic molecules from water to organic solvent that the intrinsic enthalpy of binding ($\Delta H°_i$) would have a value near zero and the intrinsic entropy of binding ($\Delta S°_i$) a value of approximately +15 eu (Jencks, 1969b; Tanford, 1973). These values for $\Delta H°_i$ and $\Delta S°_i$ are similar to the $\Delta H°_{obsd}$ and $\Delta S°_{obsd}$ found for binding to ACht (Table II). The results in Table IV then indicate that substrate-enzyme activation, ES $\rightarrow$ ES_A, is characterized by an enthalpy ($\Delta H°_a$) of approximately −10 kcal/mol and an entropy ($\Delta S°_a$) of approximately −30 eu. As these values of $\Delta H°_a$ and $\Delta S°_a$ are of the same sign, they are compensating and the $\Delta G°_a$ according to eq 7 contributes little to the $\Delta G°_{obsd}$. Accordingly, the $\Delta G°_{obsd}$ is similar to that observed in the absence of substrate-enzyme activation. However, due to the negative $\Delta S°_a$ gained in the $\Delta G°_a$ on binding to native Cht (and paid for by the favorable (negative) $\Delta H°_a$ term), the productive Michaelis complex (ES_A) in its solvent system is more "ordered" relative to association complexes without negative $\Delta H°_a$ and $\Delta S°_a$ terms. If this negative entropy found for productive type associations to native Cht can be utilized to directly lower the entropy of activation for succeeding rate-determining steps in the enzyme's catalytic mechanism, then the acceleration can be quite large. Accordingly, the changes in enthalpy and entropy indicate that a possibly catalytically important loss of entropy occurs on substrate analogue binding to native Cht, and that the association-activation process effected by these changes in enthalpy and entropy has little observed effect on the free energy of substrate binding. If this mechanism is valid and general, it has important mechanistic and physiological consequences.

In summary, we have obtained enthalpy and entropy of association data that indicated a catalytically important substrate-induced conformational change occurs on substrate binding to S_1 of native Cht at pH 7.8. This conformation change does not occur on modification of the His-57 or the Ser-195 of the catalytic site. Furthermore, the negative changes in enthalpy and entropy characteristic of the conformational change are compensating, and therefore do not affect the observed free energy of substrate analogue binding to S_1 in native Cht.

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Enzymic Formation of Glycolate in *Chromatium.* Role of Superoxide Radical in a Transketolase-Type Mechanism†

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ABSTRACT: Chromatophores prepared from *Chromatium* exhibit a light-dependent O_2 uptake in the presence of reduced 2,6-dichlorophenolindophenol, the maximum rate observed being 10.8 μmol (mg of Bchl)$^{-1}$ h^{-1} (air-saturated condition). As it was found that the uptake of O_2 was markedly inhibited by superoxide dismutase, it is suggested that molecular oxygen is subject to light-dependent monovalent reduction, resulting in the formation of the superoxide anion radical (O_2^-). By coupling baker's yeast transketolase with illuminated chromatophore preparations, it was demonstrated that [U-^{14}C]-fructose 6-phosphate (6-P) is oxidatively split to produce glycolate, and that the reaction was markedly inhibited by superoxide dismutase and less strongly by catalase. A coupled system containing yeast transketolase and xanthine plus xanthine oxidase showed a similar oxidative formation of glycolate from [U-^{14}C]fructose 6-P. It is thus suggested that photogenerated O_2^- serves as an oxidant in the transketolase-catalyzed formation of glycolate from the α,β-dihydroxyethyl (C_2) thiamine pyrophosphate complex, whereas H_2O_2 is not an efficient oxidant. The rate of glycolate formation in vitro utilizing O_2^- does not account for the in vivo rate of glycolate photosynthesis in *Chromatium* cells exposed to an O_2 atmosphere (10 μmol (mg of Bchl)$^{-1}$ h^{-1}). However, the enhancement of glycolate formation by the autoxidizable electron acceptor methyl viologen in *Chromatium* cells in O_2, as well as the strong suppression by 1,2-dihydroxybenzene-3,5-disulfonic acid (Tiron), an O_2^- scavenger, suggest that O_2^- is involved in the light-dependent formation of glycolate in vivo.

It has long been recognized that higher plants and green algae evolve O_2 photosynthetically, but at the same time they fix atmospheric O_2 in light. The mechanism of the latter phenomenon or photorespiration is one of the central problems of modern plant biology (Goldsworthy, 1970; Jackson and Volk, 1970; Chollet and Ogren, 1975). The light-induced O_2 uptake is partly explained by the RuP_2[1] oxygenase reaction which results in the formation of glycolate and the subsequent oxidation of glycolate catalyzed by glycolate oxidase (Tolbert, 1973; Glidewell and Raven, 1976). However, this enzymic mechanism alone cannot account for the overall mechanism of O_2 uptake in light (Radmer and Kok, 1976). Indeed a sizable amount of O_2 absorbed by plants appears to be ascribed to a Mehler-type reaction, and several investigators have suggested that ATP formation is coupled to this reaction (Egneus et al., 1975). It has been known that O_2 generated in the Mehler reaction is subject to a monovalent reduction process, producing O_2^- in photosystem I of chloroplast preparations (Allen and Hall, 1974; Asada et al., 1974). However, O_2^- and other reduced oxygen derivatives are known to be toxic in biological systems and it is often argued that the ubiquitous distribution of superoxide dismutase from strict anaerobic organisms to aerobes presumably reflects the detoxification of such harmful agents (Fridovich, 1975; Hewitt and Morris, 1975).

Current biochemical studies on the enzymic mechanism of glycolate formation during photosynthesis have focused on the role of O_2. A recent fashionable concept is that the oxygenase reaction catalyzed by chloroplastic RuP_2 carboxylase is responsible for the photorespiratory production of glycolate

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[1] Abbreviations used are: Bchl, bacteriochlorophyll; DCPIP, 2,6-dichlorophenolindophenol; Fd, ferredoxin; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; RuP_2, ribulose 1,5-bisphosphate; Tiron, 1,2-dihydroxybenzene-3,5-disulfonic acid; C_2, α,β-dihydroxyethyl; TPP, thiamine pyrophosphate; Tricine, *N*-tris(hydroxymethyl)methylglycine.