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Nature of the Active Site of a Subunit of the First Component of Human Complement*

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ABSTRACT: A highly purified subunit of human complement ($C1\bar{s}$) was found to hydrolyze the amino acid ester, *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester. The K_m of *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester for the purified enzyme was 5.6×10^{-5} M, the V_{max} was 2.19×10^{-6} mmole/min, and the k_{cat} was 1.22 sec^{-1}. The reaction was inhibited competitively by a variety of guanidine, amidine, and aromatic compounds of low molecular weight. The results indicated that the active center of human $C1\bar{s}$ consists of an anionic binding site in conjunction with a hydrophobic binding site.

The serum complement system of proteins has been well documented as a group of interacting proteins some of which may exhibit enzymatic properties (Müller-Eberhard, 1967). This conclusion was based on studies of the interaction of purified components of complement as well as the behavior of the intermediate complement complexes with antibody-sensitized sheep erythrocytes (Müller-Eberhard, 1967; Nelson, 1965; Mayer, 1961).

The detailed enzymatic nature of the purified complement proteins, however, has only begun to be investigated. For example, it has been known for some time that the first component of human and guinea pig complement contains an esteratic activity for synthetic amino acid esters which is inseparable from its activity in hemolysis of EA[1] (Becker, 1956; Lepow *et al.*, 1956), and that this esterase enzyme, termed $C\bar{1}$ for guinea pig complement and $C1\bar{s}$ for the human complement, probably has an anionic binding site not unlike that of trypsin (Becker, 1965).

It has also been shown that $C\bar{1}$ exists in both human and guinea pig serum in the form of a proenzyme (Lepow *et al.*, 1965; Borsos and Rapp, 1963). In the human system C1 is made up of three components, C1q, C1r, C1s; C1r converts C1s into the active enzyme $C1\bar{s}$ (Naff and Ratnoff, 1968). Recently, Westfall *et al.* (1969) have shown that butanol, methyl Cellosolve, methanol, and ethanol can noncompetitively inhibit $C1\bar{s}$. On the other hand, little is understood about the manner in which C1s generates a specificity for C2 and C4, or the exact chemical nature of the reaction of C1s with these components.

As a first step toward defining on a chemical basis the reaction of $C1\bar{s}$ with other complement components, it was decided first to examine more carefully some of the parameters involved in the binding of synthetic amino acid ester substrates to this enzyme. Analysis of the inhibition kinetics of trypsin and chymotrypsin with various types of charged and noncharged low molecular weight competitive inhibitors has led to the development of a concept of the chemical nature of the active site of these proteases, thus explaining how these enzymes generate a specificity for given amino acids in a polypeptide chain (Niemann, 1964; Mares-Guia and Shaw, 1965; Baker, 1967). This approach was used in this study to obtain similar data on human $C1\bar{s}$, namely (a) the development of a rapid spectrophotometric assay for $C1\bar{s}$ using the substrate *N*-Z-L-Tyr-*p*-Np, and (b) direct evidence for an anionic binding site in human $C1\bar{s}$.

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[1] Terminology for the complement system is that suggested in the *Bull. World Health Organ. 39*, 935 (1968). Thus $C1^-$ is the enzymatically active form of C1s, the third subunit of the first component (C1) of human complement. The other subunits of C1 are C1q and C1r. C2 and C4 are the second and fourth components of complement. EA, sheep erythrocytes treated with antisheep erythrocyte antiserum; $C\bar{1}$ inactivator is the naturally occurring serum inhibitor of $C1\bar{s}$.

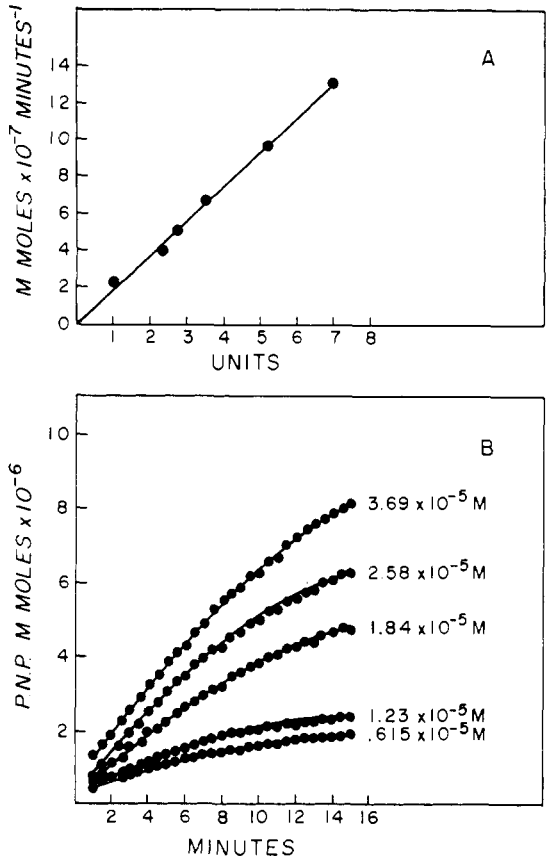


FIGURE 1: *N*-Z-L-Tyr-*p*-Np studies. (A) Hydrolysis by $\overline{Cls}$. Various concentrations of enzyme were mixed with 5×10^{-5} M *N*-Z-L-Tyr-*p*-Np in a final volume of 1 ml in 0.09 M NaCl–0.005 μM Tris-HCl (pH 8.05) and the change in optical density measured every 30 sec for 5 min. The rates were corrected for spontaneous hydrolysis and calculated using the linear portion of the curves during the first 4 min. *p*-Nitrophenol concentrations were calculated as described in Materials and Methods. (B) Extent of reaction of $\overline{Cls}$ at various N-Z-L-Tyr-*p*-Np concentrations. Each assay used 5 units of enzyme in a final volume of 1 ml in 0.09 M NaCl–0.005 μM Tris-HCl (pH 8.05). *p*-Nitrophenol concentrations were calculated as described in Materials and Methods.

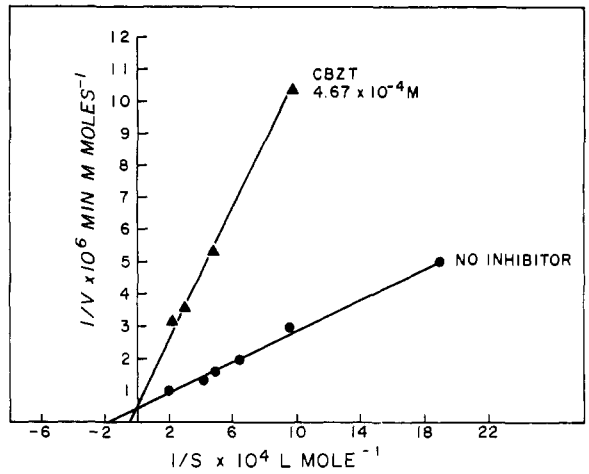


FIGURE 2: Lineweaver–Burk plot of reaction of *N*-Z-L-Tyr-*p*-Np with $\overline{Cls}$ and *N*-Z-L-Tyr inhibition of reaction of *N*-Z-L-Tyr-*p*-Np with Cls. Assays were performed and rates were calculated as described in Results. (●) Reaction of Cls in the absence of *N*-Z-L-Tyr. (▲) Reaction of $\overline{Cls}$ with *N*-Z-L-Tyr-*p*-Np in the presence of 4.67×10^{-4} M *N*-Z-L-Tyr. Ordinate, reciprocal of initial rate in min mmole^{-1}; abscissa, reciprocal of concentration of substrate in l. mole^{-1}. $V_{max} = 2.19 \times 10^{-6}$ mmole/min, $K_m = 5.16 \times 10^{-5}$ M, and $k_{cat} = 1.22$ sec^{-1}.

Materials and Methods

Proteins. Highly purified $\overline{Cls}$ was provided by Dr. I. H. Lepow, Department of Pathology, Connecticut Health Center, Hartford, Conn. It was prepared according to previously described procedures (Haines and Lepow, 1964). It had a specific activity of 502 units/mg of protein when assayed with *N*-AcTyrEt (Levy and Lepow, 1959) and a specific activity of 726 units/mg of protein when assayed with *N*-Z-L-Tyr-*p*-Np. $\overline{Cls}$ protein concentrations were determined using an $E^{1\%}_{280}$ of 10 (Nagaki and Stroud, 1969). Partially purified $\overline{C1}$ inactivator was donated by Dr. J. Pensky, Department of Pathology, Case Western Reserve University School of Medicine. It was dissolved to contain 16.6 units/ml, where 1 unit of inhibitor is defined as the amount of protein required to inhibit completely 10 units of Cls according to assay with *N*-Z-L-Try-*p*-Np.

Organic Compounds. *N*-Z-L-Tyr-*p*-Np and *N*-Z-L-Tyr were obtained from Nutritional Biochemical Corp. *N*-Z-L-Tyr-*p*-Np was determined to be greater then 99.5% homogeneous, as indicated by the presence of less than 0.4–0.39% *p*-nitrophenol in 10^{-3} M solutions of *N*-Z-L-Tyr-*p*-Np. The compounds 2-guanidine acetic acid, aminoguanidine-HCO_3, and 2,2′-dimethyl-1-guanidinopropane-HCl were obtained from Aldrich Chemical Co. *N*-Chloroacetyl-L-tyrosine was obtained from Mann Research Biochemicals. Benzamidine-HCl was obtained from K & K Fine Chemicals. Phenylguanidine sulfate and *p*-nitrobenzamidine-HCl were prepared according to previously described procedures (Smith, 1929; Pinner and Gradenwitz, 1897). Both compounds were recrystallized to constant melting points; the *p*-nitrobenzamidine had mp 299–301° (lit. mp 290° (Pinner and Gradenwitz, 1897)). The infrared spectrum (KBr pellet) indicated ν_{max} (cm^{-1}) 1675 (amidine C=N$^+$) and 1560 and 1340 (aromatic CNO_2). The phenylguanidine had a melting point of 283–284°. Infrared spectrum (KBr pellet) indicated ν_{max} (cm^{-1}) 1675 (C=NH, imine) and 1520 (C=NH, imine). DFP was a gift of Dr. H. L. Sadoff, Department of Microbiology and Public Health, Michigan State University, E. Lansing, Mich.

Enzymatic Analysis. All readings were made in a Hitachi-Coleman ultraviolet–visible 101 spectrophotometer. A stock solution of 1×10^{-3} M *N*-Z-L-Tyr-*p*-Np was made in reagent acetone and 10–50 μl of *N*-Z-L-Tyr-*p*-Np was added with a Hamilton syringe to 1 ml of 0.09 M NaCl–0.005 μM Tris-HCl buffer (pH 8.05) (Chase, 1968), containing 1–10 μg of protein. The production of *p*-nitrophenol was measured at 410 mμ by taking optical density readings every 30 sec for a period of 6–10 min. A blank containing no protein was run every time to correct for spontaneous rate of hydrolysis of *N*-Z-L-Tyr-*p*-Np. The concentration of acetone was never greater than 5% in any assay. Acetone had no effect on the activity of the enzyme between the concentrations of 0.1 and 5%. All analyses were done at 25–26°. One unit of Cls has been defined as the amount of protein which causes production of 1×10^{-6} mmole of *p*-nitrophenol in 5 min at a *N*-Z-L-Tyr-

TABLE I: Effect of Ca^{2+} and Mg^{2+} on Rate of Hydrolysis of *N*-Z-L-Tyr-*p*-Np by Human $C1\bar{s}$.[a]

$CaCl_2 \times 10^{-3}$ M	Rate (mmoles $\times 10^{-7}$/min)
2.97	4.21
2.38	5.11
1.79	5.40
1.19	5.30
0	5.45
	Rate
$MgCl_2 \times 10^{-3}$ M	Rate (mmoles $\times 10^{-7}$/min)
9.90	5.41
7194	5.23
5.96	5.23
3.98	5.30
1.995	5.34
0	5.45

[a] Enzyme concentration was 2.71 units/ml and *N*-Z-L-Tyr-*p*-Np was 3.81×10^{-5} M. Rates were calculated using linear proportion of the curve as described in Results.

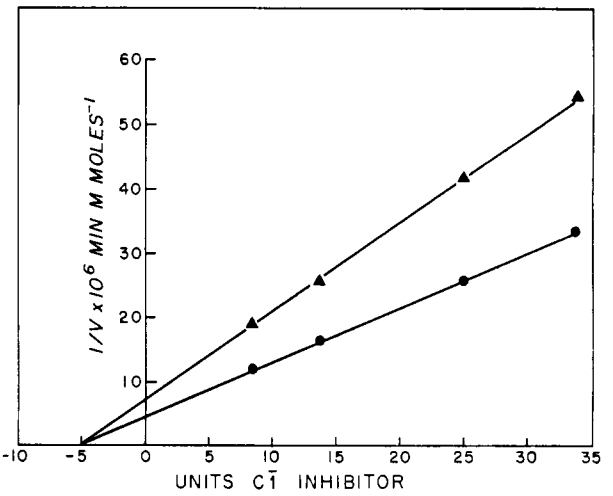


FIGURE 3: Inhibitionof $C1\bar{s}$ by serum $C\bar{1}$ inactivator. Each assay contained 4.7 units of enzyme, various amounts of inhibitor, and *N*-Z-L-Tyr-*p*-Np, in a final volume of 1 ml, in 0.09 M NaCl–0.005 μM Tris-HCl (pH 8.05). Rates were calculated as described in Results. (●) *N*-Z-L-Tyr-*p*-Np 3.1×10^{-5} M, (▲) *N*-Z-L-Tyr-*p*-Np 2.07×10^{-5} M. Ordinate, reciprocal of initial rate in min $mmole^{-1}$; abscissa, concentration of inhibitor in units/ml.

p-Np concentration of 3×10^{-4} M. The molar extinction of *p*-nitrophenol at pH 8.05 was taken as 1.66×10^4 (Chase and Shaw, 1967).

Triple-distilled water and reagent grade salts and solvents were used for making all solutions and compounds.

Results

Reaction of $C1\bar{s}$ with N-Z-L-Tyr-p-Np. The rate of hydrolysis of 5×10^{-5} M *N*-Z-L-Tyr-*p*-Np was proportional to the concentration of enzyme (Figure 1A) from 1 to 8 units of enzyme (1.38–11.1 μg of protein). Higher concentrations of *N*-Z-Tyr-*p*-Np were not tried, as it is insoluble in 0.09 M NaCl–0.005 μM Tris-HCl buffer (pH 8.05) at concentrations in excess of 6×10^{-5} M. The rate of hydrolysis of *N*-Z-L-Tyr-*p*-Np by $C1\bar{s}$ is linear for 6 min (Figure 1B). In Figure 2 is a Lineweaver–Burk plot of the rates of reaction that were calculated from data shown in Figure 1B using the linear portion of the curves during the first 4 min of reaction. Also shown is the effect of *N*-Z-L-Tyr on the rate of reaction; it can be seen that *N*-Z-L-Tyr, one product of hydrolysis of *N*-Z-L-Tyr-*p*-Np, is a competitive inhibitor of the reaction of $C1\bar{s}$ with *N*-Z-L-Tyr-*p*-Np. Thus, the decrease in rate of hydrolysis of *N*-Z-L-Tyr-*p*-Np beyond 6 min may reflect inhibition by product. The K_m for *N*-Z-L-Tyr-*p*-Np exhibited by this lot of enzyme was 5.16×10^{-5} M. V_{max} for *N*-Z-L-Tyr-*p*-Np was determined to be 2.19×10^{-6} mmole/min and k_{cat} was calculated to be 1.22 sec^{-1}, assuming a molecular weight for $C1\bar{s}$ of 80,000 (H. Müller-Eberhard, personal communication 1969). The K_i for *N*-Z-L-Tyr was determined to be 3.71×10^{-4} M. Neither Ca^{2+} nor Mg^{2+}, divalent cations which are obligate requirements for activity of the first and second components of complement in the hemolysis of antibody-treated sheep erythrocytes, had any effect on the rate of hydrolysis of *N*-Z-L-Tyr-*p*-Np by $C1\bar{s}$ (Table I). The range of concentrations of the ions examined were in the region of the concentrations of the ions required for optimal activity for hemolysis (Mayer, 1961). Finally, several other nitrophenyl esters were examined for reaction with $C1\bar{s}$. The only other compound found to be active was *N*-Z-L-Phe-*p*-Np (Table II). It however was hydrolyzed about one-half the extent of *N*-Z-L-Tyr-*p*-Np. *N*-Z-L-Ala-*p*-Np was hydrolyzed about $^1/_{16}$ the extent of *N*-Z-L-Tyr-*p*-Np.

Inhibition of $C1\bar{s}$ with $C\bar{1}$ Inactivator and DFP. The reaction of $C1\bar{s}$ with *N*-Z-L-Tyr-*p*-Np was inhibited noncompetitively by the naturally occurring serum inhibitor of $C1\bar{s}$ (Figure 3). These experiments were done by mixing various amounts of the inhibitor with 4.67 units of enzyme in the cuvette, allowing the reaction mixture to stand 5 min, and then assaying the enzyme with *N*-Z-L-Tyr-*p*-Np. Two different concentrations of *N*-Z-L-Tyr-*p*-Np, 3.1×10^{-5} and 2.07×10^{-5} M, were

TABLE II: Specificity of $C1\bar{s}$ for *N*-Z-L-Tyr-*p*-Np.[a]

Substrate	Concn (M)	mmoles $\times 10^{-6}$ *p*-Nitrophenol 5 min	15 min
N-Z-L-Tyr	3.07×10^{-5}	3.13	5.32
N-Z-L-Phe	3.34×10^{-5}	1.81	2.71
N-Z-L-Ala	3.71×10^{-5}	0.066	0.904

[a] Enzyme concentration was 4.67 units/ml; assays were done in a final volume of 1 ml, in 0.09 M NaCl–0.005 μM Tris-HCl (pH 8.05).

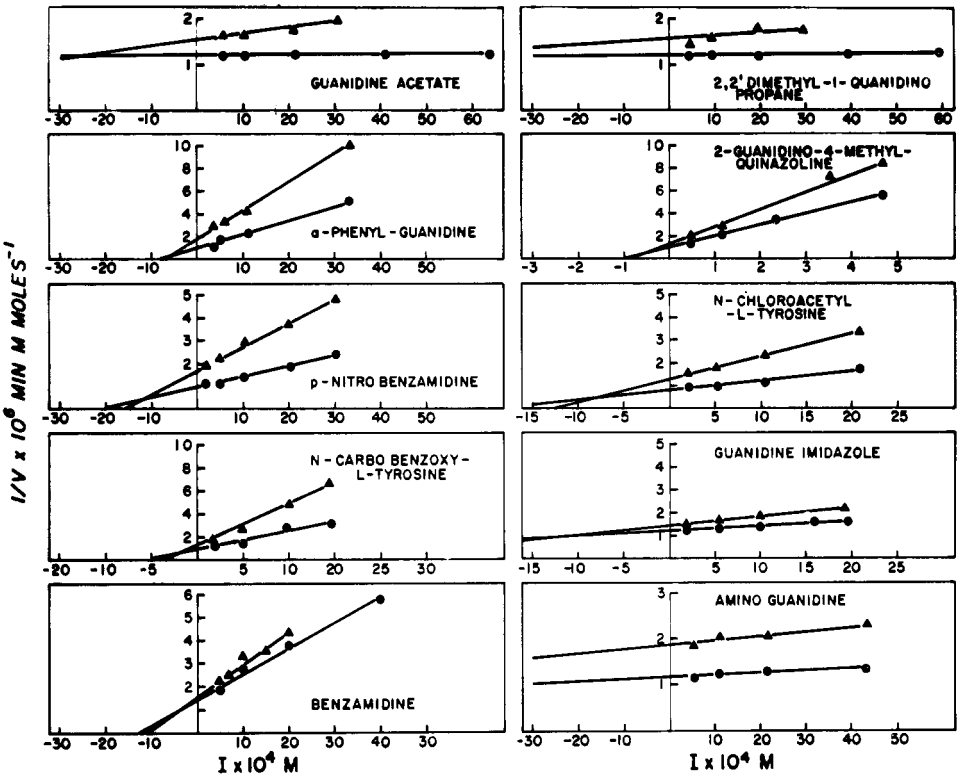


FIGURE 4: Competitive inhibition of $C1\bar{s}$ by guanidines, amidines, and tyrosine compounds. Ordinate, reciprocal of initial rate in min mmole^{-1}. Abscissa, molar concentration of inhibitors. Assays were done and rates were calculated as described in Results. (▲) 2.08×10^{-5} M *N*-Z-L-Tyr-*p*-Np, (●) 3.15×10^{-5} M *N*-Z-L-Tyr-*p*-Np. Each assay used 4.67 units of enzyme/ml.

examined at each different concentration of inactivator. The results were plotted according to the method of Dixon (1953). The intersection of the lines on the *x* axis is indicative of noncompetitive inhibition.

Haines and Lepow (1964) measuring hydrolysis of *N*-Ac-L-TyrEt reported that DFP inhibited the reaction of serum $C\bar{1}$ inactivator with $C1\bar{s}$, indicating that this inhibitor was acting near the active site of $C1\bar{s}$. Similar experiments using *N*-Z-L-Tyr-*p*-Np for assay gave identical results.

$C1\bar{s}$ was treated with 10^{-2} M DFP for 10 min at 37° at a concentration of 30 units of enzyme/ml in a volume of 0.5 ml. The DFP-treated protein and a control, which was incubated at 37° for 10 min without DFP, were then dialyzed 5 hr against 0.09 M NaCl–0.005 μM Tris-HCl (pH 8.05). Then 0.1 ml of each was mixed with $C\bar{1}$ inactivator and assayed either for free enzyme in the case of the control, or for free inhibitor in the case of the DFP-treated $C1\bar{s}$. In the latter case, the presence of free inhibitor was determined by adding 4.75 units of untreated enzyme before assaying with 3×10^{-5} M *N*-Z-L-Tyr-*p*-Np. As seen in Table III, close to theoretical amounts of free enzyme or free $C\bar{1}$ inactivator were found in each case. These two experiments indicated that *N*-Z-L-Tyr-*p*-Np was measuring the same esteratic activity of $C1\bar{s}$ as is measured with *N*-Ac-L-TyrEt.

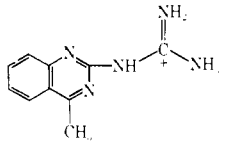
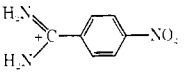
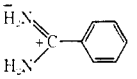
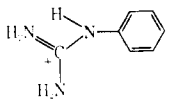
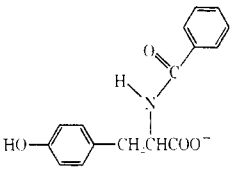
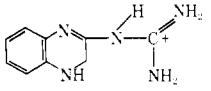
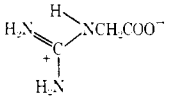
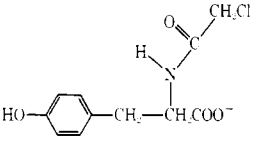
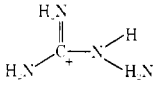
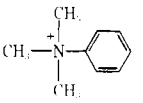
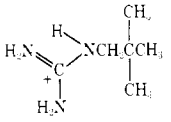
Competitive Inhibition of $C1\bar{s}$. In order to gain some insight into the nature of the esteratic site of $C1\bar{s}$, a series of compounds were examined as inhibitors of the reaction with *N*-Z-L-Tyr-*p*-Np. Inhibition was measured at two concentrations of substrate, 2.08×10^{-5} and 3.15×10^{-5} M, using 4.67 units of enzyme for each assay. Inhibitor concentration was varied such that there was between 10 and 80% inhibition. The initial rates of reaction were calculated as previously described and the results were plotted according to the method of Dixon (1953). Most of the compounds exhibited competitive inhibition, as evidenced by the intersection of the lines above the *x* axis. Inhibition constants were calculated using the slopes and the previously determined V_{max} (Dixon, 1953).

TABLE III: Effect of DFP on Binding of Serum $C\bar{1}$ Inactivator by $C1\bar{s}$.[a]

Control[b]	Units of Inactivator/ml (Added)	Free Enzyme (Obsd)	Theor Free Enzyme
2.76	0.000	2.76	2.76
2.76	0.166	1.93	1.91
2.76	0.249	1.27	1.49
$C1\bar{s}$ Treated with DFP[c]	**Units of Inactivator/ml (Added)**	**Free Inactivator/ml (Obsd)**	
0.042	0.000	0.000	
0.042	0.085	0.085	
0.042	0.132	0.134	

[a] See Results for experimental details. [b] $C1\bar{s}$ added in units per milliliter. [c] In units per milliliter,

TABLE IV: Competitive Inhibitors of C1s.[a]

Inhibitor	K_i (M)	$\Delta F'$ (kcal/mole)	Inhibitor	K_i(m)	$\Delta F'$ (kcal/mole)
2-Guanidino-4-methylquinazoline 	7.65×10^{-5}	4.95	*p*-Nitrobenzamidine 	9.25×10^{-2}	2.25
			Benzamidine 	6.03×10^{-4}	3.87
α-Phenylguanidine 	5.48×10^{-4}	3.92			
			N-α-L-Tyr 	3.7×10^{-4}	4.13
2-Guanidinobenzimidazole 	2.97×10^{-3}	3.04			
Guanidineacetic 	4.37×10^{-3}	2.86	*N*-Chloroacetyl-L-tyrosine 	1.12×10^{-3}	3.55
Aminoguanidine 	1.81×10^{-2}	2.09	Trimethylphenylammonium 		No inhibition at 2.5×10^{-3} M
2,2'-Dimethyl-1-guanidinopropane 	1.34×10^{-2}	2.25			

[a] K_i's were calculated using the slopes and calculated V_{max}. $\Delta F' = -2.303RT \log K_i$. Experiments were performed at 25° and at pH 8.05.

The K_i values, along with the $\Delta F'$[2] values calculated from the K_i values, are given in Table IV. The K_i's ranged from 7.65×10^{-5} M for 2-guanidino-4-methylquinazoline to 1.3×10^{-2} M for 2,2'-dimethyl-1-guanidinopropane, and $\Delta F'$ ranged from 4.95 to 2.25 kcal/mole.

Discussion

N-Z-L-Tyr-*p*-Np, first described by Martin *et al.* (1958), has been used for the assay of both chymotrypsin and trypsin. The extent of reaction of these enzymes with this substrate is determined by measuring the release of *p*-nitrophenol spectrophotometrically at pH 8.0. The substrate is extremely sensitive; nanogram amounts of enzyme can be measured with *N*-Z-L-Tyr-*p*-Np, and the reaction only takes a few minutes. A human C1s behaves similarly with *N*-Z-L-Tyr-*p*-Np; there is a linear rate of hydrolysis of *N*-Z-L-Tyr-*p*-Np up to about 6 min. In the case of the C1s this decrease in rate probably is due to end-product inhibition, as *N*-Z-L-Tyr, one product of the reaction, is a potent competitive inhibitor with a $K_i = 3.71 \times 10^{-4}$ M. As with trypsin and chymotrypsin, the rate of hydrolysis of *N*-Z-L-Tyr-*p*-Np by C1s is proportional to enzyme concentration. The reaction of C1s with *N*-Z-L-Tyr-*p*-Np is dissimilar from trypsin and chymotrypsin in terms of the amount of protein required and divalent cation requirement; microgram amounts of the complement enzyme are required in contrast to the nanogram amounts of trypsin and chymotrypsin employed, and neither Ca^{2+} nor Mg^{2+} has any effect on the reaction of C1s whereas Ca^{2+} is required to stabilize the trypsin and chymotrypsin solutions.

[2] $\Delta F'$ was calculated using the relationship $\Delta F' = -RT \ln K_i$. The K_i is a measure of affinity of the enzyme for the inhibitor (Dixon and Webb, 1958). $\Delta F'$ has been used instead of $\Delta F°$ since the latter term (or $\Delta G°$) is usually reserved for the absolute free energy of a process where standard states are completely defined.

TABLE V: Contribution of Phenyl and Acetate Moieties of Respective Guanidines to Binding to $C1\bar{s}$.[a]

Group	$\Delta(\Delta F')$, kcal/mole		Interaction on One Side (Calcd) (kcal/mole)	Interaction on Two Sides (Calcd) (kcal/mole)	Difference between Phenyl and Acetate (kcal/mole)
	Obsd	Cor			
Phenyl	1.92	2.44	2.35	4.70	1.15[b]
Acetate	0.77	1.29	1.36	2.72	0.99[c]

[a] Calculations were made as described in the Discussion. [b] Observed. [c] Calculated.

It was concluded that *N*-Z-L-Tyr-*p*-Np was detecting the identical esterase activity of $C1\bar{s}$ measured with *N*-Ac-L-TyrEt, based on the studies with the naturally occurring serum $C\bar{1}$ inactivator and the acylating reagent, DFP. The serum inactivator inhibited the reaction with *N*-Z-L-Tyr-*p*-Np noncompetitively as would be predicted based on the nature of the reaction of this inhibitor with $C1\bar{s}$; this inhibitor binds stoichiometrically with $C1\bar{s}$ to inhibit all of the phenomena known to be mediated by this component of complement (Lepow *et al.*, 1965). Haines and Lepow (1964) previously demonstrated that DFP simultaneously destroyed esterase activity toward *N*-Ac-L-TyrEt and the binding of the serum inhibitor. Similar experiments in this study gave identical results. DFP-inactivated $C1\bar{s}$ bound no serum inhibitor according to the assay with *N*-Z-L-Tyr-*p*-Np.

The assay of purified $C1\bar{s}$ with *N*-Z-L-Tyr-*p*-Np was judged to be somewhat more simple as compared to the assay with *N*-Ac-L-TyrEt. The reaction could be done with microgram amounts of protein in 1-ml volumes, and the reaction could be terminated after 5 min at 25°. It also appeared that *N*-Z-L-Tyr-*p*-Np was a much more sensitive substrate for the measurement of human $C1\bar{s}$. Besides the fact that 10^{-5} M concentrations of substrate were sufficient to detect the enzyme (in contrast to 10^{-3} M concentrations of substrate used in the assay with *N*-Ac-L-TyrEt) (Haines and Lepow, 1964), it was noted that the specific activity of $C1\bar{s}$ was always about 1.5 times greater according to the assay with *N*-Z-L-Tyr-*p*-Np than the specific activity determined with *N*-Ac-L-TyrEt.

The development of this assay permitted the rapid screening of a number of compounds as potential inhibitors. In general, it was found that a variety of aromatic guanidine and amidine compounds could be competitive inhibitors of the enzyme. The data on the guanidines will be considered first. Phenylguanidine, besides being similar in conformation, has approximately the same length as the side chain of Arg or Lys from the nucleus of the β-carbon atom to the positive group when the amino acids are held fully extended and the positive charges are coincident (Mares-Guia and Shaw, 1965). Phenylguanidine, while having the characteristics of a planar molecule, is not symmetrical about the axis which bisects the phenyl ring. This molecule, therefore, probably represents a good model for the side chains of amino acids.

If aminoguanidine is taken as a reference it is possible to calculate the relative contribution to binding made by the phenyl ring.

$$\Delta F'(\text{phenylguanidine}) - \Delta F'(\text{aminoguanidine}) = \Delta(\Delta F')\ C_6H_6 - NH_2 = 1.92 \text{ kcal/mole}$$

These calculations are tabulated in Table V. It should be noted at this point that the $\Delta F'$'s were calculated using the K_i which is a dissociation constant (Dixon and Webb, 1958). Thus the $\Delta F'$ will have a positive rather than a negative sign as would be the case in situations where an association constant is measured. If it is assumed that contributions of the phenyl ring and acetate side chains are due to London dispersion forces, a very good agreement is obtained between the observed data and calculated values for interactions of such groups with proteins.

London dispersion forces represent the principle force of attraction between neutral molecules. These forces were first quantitatively described by London (1937) and may be summarized as follows. In effect, a neutral molecule can become polarized due to fluctuations in the nuclei and outer electrons. The net result of this dipole is the polarization of a second molecule, and the distribution of the electrons will continuously favor attraction of both molecules (London, 1937). Webb (1963) has calculated dispersion interaction energies for various substituents reacting with a protein surface using equations previously described by Pauling and Pressman (1945) for describing reactions of hapten and antihapten antibodies. According to such calculations, the calculated dispersion energy for a benzene ring interacting with one side of a protein is 2.35 kcal/mole and for an acetate is 1.36 kcal/mole (Webb, 1963). The $\Delta(\Delta F')$ values in the present work were interpreted to be the difference in binding between phenyl or acetate and an amino group, as aminoguanidine was used as a reference. To obtain the contributions of the phenyl and acetate groups relative to hydrogen, the calculated values have to be corrected by adding the energy of the dispersion interaction for the amino group to the observed values for the $\Delta(\Delta F')$. This was done using 0.52 kcal/mole, a figure calculated by Webb (1963) to be the dispersion energy for the interaction of amino groups with a protein surface. The values of $\Delta(\Delta F')$ calculated from the experimental results (Table V) are very close to values quoted by Webb (1963) for the interaction of the groups with one side of a protein.

This discussion of London dispersion forces neglects the important contribution of the solvent in the process. Such an interpretation would necessarily neglect both entropy changes in water displacement or restructuring and hydrogen bonding between water and the interacting groups (Lumry, 1959). A more general interpretation of our results could be that the binding of the inhibitors occurs through the formation of hydrophobic bonds with the side chains carrying polar groups. If the interaction with the inhibitor is viewed as a partial transfer of the inhibitor from an aqueous environment to a

nonpolar environment on the surface of the protein, then it is possible to compare contribution of the methylene group of acetate with the energy release involved in a methylene–methylene interaction. The process of hydrophobic bonding has a maximum release of 700 cal/mole for $>CH_2-CH_2<$ interaction (Baker, 1967). If this value is compared with the uncorrected $\Delta F'$ value for acetate, there is remarkably good agreement between this value and the observed 770 cal/mole.

These considerations and the good agreement with calculated and observed values are taken to mean that the side chains of these inhibitors form hydrophobic bonds with a readily available (perhaps exposed) concavity on the surface of C1s and this site must be adjacent if not continuous with an anionic binding site to which the guanidinium group can bind through its positive charge. Furthermore, this site evidently has somewhat different properties from the hydrophobic crevice with an anionic binding site which has been described for trypsin (Mares-Guia and Shaw, 1965); first, trimethylammonium does not inhibit, so there has to be more than just a positive charge in conjunction with an aromatic ring to obtain binding. This is in direct contrast to trypsin (Ingami, 1964). Second, the $\Delta F'$ (uncorrected) (1.92 kcal/mole) for the phenyl group is only about half of the $\Delta F'$ for solution of benzene in water (4.61 kcal/mole) indicating perhaps only partial transfer of the phenyl ring into a nonaqueous environment. This is further supported by the less than theroetical $\Delta F'$ observed for 2-guanidino-4-methylquinazoline (if it were to be transferred entirely to a nonaqueous environment). Third, the hydrophobic site must be larger than just one phenyl ring. The $\Delta F'$ for the 2-guanidino-4-methylquinazoline is almost 1 kcal/mole more than for phenylguanidine suggesting the existence of additional structure(s) which can permit a greater degree of hydrophobic bonding. This idea is further supported in that this same kind of difference in $\Delta F'$ is seen between *N*-carbobenzoxy-L-tyrosine and *N*-chloroacetyltyrosine, which also differ by one benzene ring. Fourth, the entire anionic–hydrophobic site must be reasonably flexible, as the benzamidine compounds are as good inhibitors as phenylguanidine. Benzamidine is similar to phenylguanidine except there is one less amino group and the phenyl ring will thus be somewhat displaced in relation to the positive charge as compared with phenylguanidine. On the other hand, if the phenyl ring is displaced too far from the hydrophobic binding site there is less than optimal binding. The lower binding of guanidinobenzimidazole as compared with phenylguanidine would be explained by such a situation. Here, the imidazole ring is displacing the benzene ring and the inhibitor cannot be as readily accommodated by the site. The same interpretation would explain the low binding of the 2,2′-dimethylpropyl group, where the hydrophobic group would also be poorly bound.

The fact that an enzyme with an apparent anionic binding site can hydrolyze a noncharged substrate deserves some comment. It should be recalled that this is not unprecedented, as trypsin, an enzyme with an anionic binding site, does hydrolyze *N*-Z-L-Tyr-*p*-Np quite readily (Martin *et al.*, 1958), and can bind neutral molecules (Baker, 1967). An enzyme site, such as that of C1s, containing more than one parameter which can influence binding of a substrate or inhibitor means that there can be more than one kind of structure which can bind. In the case of *N*-Z-L-Tyr-*p*-Np or *N*-Z-L-Tyr, the aromatic rings contribute most to the binding, and the hydroxyl, while probably contributing to the energy of interaction, is not of primary importance in determining binding of the particular compound by the enzyme. In contrast, in the case of the guanidine or amidine group, the positive charge makes possible cooperative ion–ion interactions and contributes significantly to the over-all binding. Currently, experiments are being designed to test further this point and other concepts brought out in this discussion.

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Isolation and Characterization of Polypeptides of Human Serum Lipoproteins*

B. Shore and V. Shore

ABSTRACT: Two or more different polypeptides were isolated by DEAE-cellulose chromatography from each of the protein moieties of several fractions of human serum lipoproteins. The polypeptides were characterized by amino acid composition, carboxyl-terminal analysis, and polyacrylamide gel electrophoresis. The high-density lipoproteins of density 1.083–1.124 g/cc (HDL_2) were more heterogeneous than those of density 1.126–1.195 g/cc (HDL_3) with respect to polypeptide content and contained several polypeptides in addition to the two which comprise most of the protein of the HDL_3 fraction.

The low-density lipoprotein fraction of density 0.98–1.006 g/cc (S_f 20–100) also contains several polypeptides, two of which are similar to if not identical with peptides found as minor components in the high-density lipoproteins. The protein of the low-density lipoprotein fraction of density 1.029–1.039 g/cc (S_f 4–8 lipoproteins) yielded two polypeptides, which were different from the peptides of high-density lipoproteins and the S_f 20–100 fraction of low-density lipoproteins. Multiple forms, differing slightly in amino acid composition, of some of the peptides were found. The high-density lipoproteins and the low-density lipoproteins may be structurally and metabolically related by their content of lipid complexes of two polypeptides, one of which has carboxyl-terminal R-Ala-Val-Ala-Ala and one of which has an unusually high content of glycine, serine, and glutamic acid, which are major components of S_f 20–100 lipoproteins and minor components of HDL_2 lipoproteins.

The high-density lipoproteins of human serum contain two different polypeptides which have been isolated and characterized with respect to molecular weight, amino acid composition, and carboxyl-terminal sequence (Shore and Shore, 1968a,b). In the present work, the study is extended to the minor components of high-density lipoproteins (HDL)[1] and the proteins of low-density lipoproteins. Possible structural and metabolic relationships between high-density lipoproteins and low-density lipoproteins were explored on the basis of polypeptide composition of the protein moieties.

In this paper, we report the isolation and composition of several minor polypeptide components of HDL_2 (1.083–1.124 g/cc of lipoproteins) in addition to the two major ones previously isolated from the denser HDL_3 (1.126–1.195 g/cc) lipoproteins, which are less heterogeneous with respect to polypeptides. The protein moiety of S_f[2] 4–8 lipoproteins (1.029–1.039 g/cc) yielded none of the polypeptides found in HDL, but the very low-density lipoprotein yielded two polypeptides similar to if not identical in composition with two of the minor HDL components in addition to three other peptides not found in HDL or S_f 4–8 lipoproteins. Some of the polypeptides of HDL and the very low-density lipoprotein were found in multiple forms very similar but not identical in composition.

Experimental Section[3]

Materials. Human serum lipoprotein fractions were isolated from the serum of individual male and female donors, apparently healthy and nonfasting, by preparative centrifugation at 11–12° in a Spinco–Beckman Model L-2-65B centrifuge. NaEDTA (8×10^{-4} M) and Tris-HCl (0.015 M) at pH 7.4 were present at all stages of lipoprotein isolation. The fractions were characterized by density, per cent lipid, and S_f rate, which was calculated from the rate of movement of the maximum ordinate of the schlieren peak. Carbohydrates, present in small amounts in some human serum lipoproteins (Marshall and Kummerow, 1962; Scanu, 1966), were not determined. The amounts of the various lipoprotein fractions obtained from the serum samples were within the normal ranges reported by Gofman *et al.* (1954).

The S_f 20–100 lipoproteins (<1.007 g/cc) were isolated by centrifugation of 180–200 ml of serum for 36 hr at 30,000 rpm in a 30.2 rotor. The lipoproteins in the top 1-ml portions were combined, diluted to 180 ml with a sodium chloride solution

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[1] Abbreviation used is: HDL, high-density lipoprotein.

[2] S_f denotes the negative sedimentation coefficient in Svedbergs in density 1.063 g/cc NaCl solution at 26°.

[3] Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Atomic Energy Commission to the exclusion of others that may be suitable.