SHORT COMMUNICATION

# Intramolecular Hydrogen Bonding, a Factor in Benzoate-Antibenzoate Combination

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SUMMARY

In order for a substituted benzoate ion to combine most effectively with the receptor site of antibody to the *p*-azobenzoate group, it appears that the carboxylate group must be coplanar with the benzene ring. *o*-Aminobenzoate and monosubstituted *o*-aminobenzoates are accommodated, while disubstituted *o*-aminobenzoates are not. The ability of the former compounds to form an intramolecular hydrogen bond between the hydrogen of the amino group and the carboxylate preserves the planar structure which appears to be necessary for effective combination with antibody. Thus the observation that *ortho* substituents in general interfere with the combination stems from the fact that they tilt the carboxylate out of the plane of the benzene ring.

The problem of hydrogen bonding is an important one in the interaction of ligands with specific receptor sites. Not only are hydrogen bonds important as factors in the strength of the interaction, i.e., by their formation between the ligand and receptor, but they may also be important when the ligand can form an intramolecular hydrogen bond which affects the steric configuration of the ligand and stabilizes a certain structure.

The binding of *o*-aminobenzoates to the receptor sites of antibodies against the *p*-azobenzoate group (anti-$X_p$ antibodies) provides a good example of the effects produced by intramolecular hydrogen bonding. Such antibodies seem to fit closely about the positions *ortho* to the carboxylate group and bind most *ortho*-substituted benzoates only poorly (1). Thus replacement of an *ortho* hydrogen of the benzene ring by another group such as bromine, methyl, or nitro alters the structure of benzoate sufficiently to decrease appreciably the binding constant for combination with antibody. The effect presumably occurs through steric factors, either by direct interference or by tilting the carboxylate group out of the plane of the benzene ring. On the other hand, it was found that the antibody would accommodate an *ortho*-substituted benzoate when the substituent was an amino group or a monosubstituted amino such as acetylamino or phenylamino. Since in these substances the *o*-amino group has a proton which can form a hydrogen bond with the carboxylate, it was

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pointed out that such hydrogen bonding is probably responsible for the accommodation.

We have now carried out further studies using benzoates with disubstituted amino groups in the *ortho* position. The relative binding constants of these aminobenzoates were compared with the related monosubstituted ones, which still contained a hydrogen available for hydrogen bonding to the carboxylate. We have found that those substances containing a monosubstituted amino group combined much better with anti-$X_p$ antibody than did other *ortho*-substituted compounds, whereas those *o*-amino compounds in which both hydrogens have been replaced show the low combination usually observed with other *ortho*-substituted benzoates. The observations emphasize the importance of the hydrogen on the *o*-amino group for good combination.

The immunizing antigen (BGG-$X_p$) was prepared by coupling diazotized *p*-aminobenzoic acid (300 mg) with bovine γ-globulin (Pentex Fraction II) (10 g). The resultant azoprotein was dialyzed exhaustively before use. The test antigen (oval-$X_p$) was prepared by coupling the diazotized amine (61 mg) to ovalbumin (1 g), and uncoupled hapten was removed by acetone extraction or by passage through a column of Sephadex G-25.

Rabbits received 1 ml of a 1% solution of the azoprotein (BGG-$X_p$) at pH 8.0 in sodium borate buffer intravenously three times a week for 3 weeks. One week after the last injection, and weekly thereafter, the animals were bled. One milliliter of antigen solution was injected after each bleeding. The sera from several rabbits were pooled according to titer as determined by precipitation with the test antigen. One pool was used in the present investigation. The γ-globulin fraction of the pooled antiserum was prepared by the method of Kekwick (2) (three precipitations with decreasing concentrations of $Na_2SO_4$ at room temperature).

Borate buffer was prepared by dissolving 195.2 g of $H_3BO_3$, 20.2 g of NaOH, and 148.2 g of NaCl in distilled water and making the solution up to a volume of 19 liters. The pH of this buffer at 25° was 7.9–8.0.

Most haptens were commercial products and were recrystallized to the correct melting point and equivalent weight. A few that were not commercially available were prepared in the laboratory. *o*-Dimethylaminobenzoic acid was prepared by methylating sodium anthranilate with dimethyl sulfate and hydrolyzing the resultant *o*-dimethylaminobenzoic acid methyl ester. *o*-Benzoylaminobenzoic acid was prepared by benzoylation of *o*-aminobenzoic acid with an equivalent amount of benzoyl chloride in the presence of sodium hydroxide. *o*-Acetylmethylaminobenzoic acid was synthesized by acetylation of *o*-methylaminobenzoic acid with acetic acid anhydride in aqueous solution in the presence of sodium carbonate and sodium bicarbonate. *o*-Benzoylmethylaminobenzoic acid was made by benzoylation of *o*-methylaminobenzoic acid with benzoyl chloride in the aqueous solution of sodium carbonate and sodium bicarbonate.

Hapten solutions of different molarity were prepared by dissolving the required amount of hapten in a molar equivalent of 1 N NaOH and diluting with borate buffer, pH 8.0. The final pH of the hapten solution was 7.9–8.0.

The amount of test antigen required to give optimum precipitation of antibody at a fixed concentration of γ-globulin was determined in advance. Experiments on the hapten inhibition of precipitation were carried out by adding 0.5-ml portions of hapten solution, test antigen (optimum concentration), and γ-globulin to a centrifuge tube, in that order. Duplicate samples with each hapten were run at four concentrations; each of the components was at pH 8.0. The mixture was permitted to stand for 1 hr at 37° and then for 62–65 hr at 4° without disturbance. Precipitates were centrifuged and washed three times with 10-ml portions of borate buffer, pH 8.0. The amount of protein in the precipitates was determined by a modified Folin method (3). Control experiments were also carried out in duplicate, in which only antigen and antibody were present and a 0.5-ml portion

of borate buffer replaced the hapten solution. Blanks containing 1.0 ml of borate buffer and 0.5 ml of γ-globulin were run under the same conditions in order to correct for small amounts of protein that precipitated from γ-globulin solution standing at 3–5°. The same preparation of γ-globulin was used throughout.

The effects of various benzoate derivatives with substituents in the *ortho* and *para* positions on the precipitation of rabbit anti-$X_p$ antibodies with $X_p$-oval are shown in Table 1. Results were interpreted by application of a theory of heterogeneity of combining sites of antibody (4), based on the assumption of a Gaussian distribution of combining free energies, to give values of $K_{rel}$, $\sigma$, and $\Delta F_{rel}$. $K_{rel}$ is the average equilibrium constant for the reaction between hapten and antibody relative to that for the reaction between antibody and unsubstituted benzoate, the reference hapten, which is arbitrarily assigned a $K_{rel}$ value of 1.00. $\sigma$ is an index of heterogeneity, which appears as an exponent in the distribution function. The value of $\Delta F_{rel}$ represents the difference between the free energy of combination of the hapten with antibody and that of the unsubstituted benzoate with antibody, calculated for 4°.

The results obtained indicate that both

TABLE 1

*Combination of substituted benzoates with anti-p-azobenzoate antibodies (effect of hapten on precipitation of anti-$X_p$ antibodies with $X_p$-ovalbumin)*

Globulin fraction of anti-$X_p$ antiserum, 4.8 mg in 0.50 ml of borate buffer; $X_p$-oval, 0.13 mg in 0.50 ml of borate buffer; 0.5 ml of hapten in borate buffer.

| Benzoate hapten | $K_{rel}$ | $\Delta F_{rel}$ | $\sigma$ | Amount of precipitate[a] at final hapten concentrations (M × $10^4$) of: 1.0 | 2.1 | 4.1 | 8.3 | 16.7 | 33.3 | 66.7 | 133 | 267 | 533 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *cal* | | % | % | % | % | % | % | % | % | % | % |
| Unsubstituted | 1.00 | 0 | 3.0 | | 69 | | 46 | | 25 | | 12 | | |
| *o*-$NH_2$ | 0.68 | 215 | 3.0 | | 78 | | 54 | | 30 | | 19 | | |
| *o*-NHPhe | 0.60 | 280 | 2.5 | 78 | | 63 | | 47 | | 16 | | | |
| *o*-NH—C(=O)—Phe | 0.45 | 445 | 3.5 | | 75 | | 55 | | 41 | | 16 | | |
| *o*-NH—C(=O)—$CH_3$ | 0.36 | 570 | 3.5 | | 77 | | 65 | | 41 | | 21 | | |
| *o*-NH—$CH_3$ | 0.27 | 730 | 3.5 | 84 | | 74 | | 59 | | 37 | | | |
| *o*-$N(CH_3)_2$ | 0.074 | 1450 | 2.5 | | | | 88 | | 75 | | 48 | | 15 |
| *o*-N(C(=O)—Phe)($CH_3$) | 0.036 | 1710 | 2.5 | | | | 87 | | 80 | | 62 | | 33 |
| *o*-N(C(=O)—$CH_3$)($CH_3$) | 0.017 | 2120 | 2.5 | | | | 90 | | 87 | | 77 | | 50 |
| *o*-$NO_2$ | 0.083 | 1380 | 4.0 | | | 81 | | 72 | | 47 | | 34 | |
| *o*-$CH_3$ | 0.076 | 1435 | 4.0 | | | 81 | | 66 | | 50 | | 38 | |
| *p*-$N(CH_3)_2$ | 3.16 | −640 | 3.5 | 58 | | 38 | | 20 | | 13 | | | |
| *p*-$NH(CH_3)$ | 1.78 | −320 | 3.0 | 69 | | 44 | | 25 | | 11 | | | |
| *p*-$NH_2$ | 1.20 | −100 | 3.0 | 76 | | 54 | | 33 | | 14 | | | |

[a] The percentage of the amount of precipitate formed in the absence of hapten (202 μg).

*o*-methyl and *o*-nitro substituents produced an increase in $\Delta F_{rel}$ of over 1300 cal (a decrease in $K_{rel}$ to a value below 0.1), while the *o*-amino substituent produced an increase in $\Delta F_{rel}$ of only 215 cal. Replacing 1 hydrogen of the *o*-amino group by a phenyl increased $\Delta F_{rel}$ by only an additional 65 cal, and replacement by a methyl increased $\Delta F_{rel}$ by 515 cal. Replacement by benzoyl or acetyl gave intermediate values. However, replacing both hydrogens of the *o*-amino group by other groups increased $\Delta F_{rel}$ by 1235–1905 cal, to give $\Delta F_{rel}$ values comparable to those observed with the *o*-nitro or *o*-methyl substituents.

These results show the requirement of at least 1 hydrogen on the nitrogen *ortho* to the carboxylate in order to negate the generally observed effect of an *ortho* substituent to increase $\Delta F_{rel}$ greatly. Because of the large size of substituents, i.e., phenyl, benzoyl, and acetyl, which can be accommodated when the hydrogen is present, it appears that the main reason other *ortho* substituents increase $\Delta F_{rel}$ by large values is that they tilt the carboxylate out of the plane of the benzene ring rather than interfering sterically between the ligand and receptor. This tilt interferes with the combination with the receptor site directed against a carboxylate group planar with the benzene ring. The tilt does not occur when a hydrogen is available on the *ortho* nitrogen, because it forms a hydrogen bond with the carboxylate to hold it in the planar position.

An interesting corollary to this phenomenon is the ability of the antibody to accommodate the large groups in the *ortho* position. It would appear that the receptor is directed against the face of the benzoate rather than surrounding it, as would be the case with an invaginated receptor site. Thus the regions adjacent to the receptor site can accommodate large groups such as phenyl and benzoyl. Indeed, this ability to accommodate is reflected by the fact that the phenylamino group causes stronger combination than the methylamino group, and the benzoylamino group causes stronger combination than the acetylamino group. The greater combination with the phenyl group in the place of the methyl indicates that the phenyl can contribute more combining energy because of its greater polarizability and hydrophobic properties.

The *p*-amino substituents, i.e., *p*-amino, *p*-methylamino, and *p*-dimethylamino, increased the combining constant of benzoate. The dimethyl-substituted amino group caused better combination than the monosubstituted or unsubstituted amino group. This effect was the opposite of that observed with the *o*-amino group. It is evident that the *para* position of benzoate is that occupied by the azo group in the immunizing antigen (*p*-azobenzoate). The antibodies were formed to accommodate an azo group in this position, and can accommodate other substituents with a correspondingly greater combining constant than when hydrogen occupies this position.

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