J. Nutr. Sci. Vitaminol., **23**, 145–152, 1977

IDENTIFICATION AND PROPERTIES OF REACTIVE SITES IN PROTEIN CAPABLE OF BINDING CARBON DIOXIDE IN A GAS-SOLID PHASE SYSTEM[1]

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(Received September 29, 1976)

Summary In order to identify the functional groups which really contribute to the carbon dioxide gas adsorption by proteins, ε-amino groups of lysine residues of egg albumin were chemically modified with trinitrobenzene sulfonic acid to various degrees. About 60% of the total amount of carbon dioxide gas adsorbed by solid egg albumin diminished by complete modification.

The amount of carbon dioxide gas adsorbed by lysozyme, its hydrolyzates and gelatin hydrolyzates depended upon the lysine content, arginine content and average molecular weight. The good correlation was obtained between the amount of carbon dioxide gas adsorbed and the total of lysine and arginine content of them.

The ability of carbon dioxide gas adsorption by α-amino group of amino acids and oligopeptides was found to be developed by the elongation of the peptide chain of glycine and other amino acid, by the removal of α-carboxyl group of histidine and tyrosine to corresponding amines and by the esterification of α-carboxyl group of leucine with *p*-nitrophenol. These results clearly indicate that CO_2 binding sites in protein in the gas-solid phase system are ε-amino, α-amino and guanidinium groups.

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[1] The contents of this paper were presented at the annual meeting of the Food and Nutrition Society of Japan at Sendai (May, 1976).

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Abbreviations: TNBS, trinitrobenzene sulfonic acid; TNP-, trinitrophenyl-; PNP-, *p*-nitrophenyl-; Cbz-Leu-PNP ester, N-carbobenzyloxy-L-leucine-*p*-nitrophenyl ester; Leu-PNP ester, leucine-*p*-nitrophenyl ester.

It has been shown by the present authors that a significant amount of CO_2 gas is adsorbed by cereal grains and attempts were made to elucidate the mechanism involved (*1,2*). In the course of investigation, various proteins in solid form were found to be capable of adsorbing CO_2 gas. As preliminaries to this paper, the authors reported the results of packaging tests of purified protein and protein foods in the CO_2 gas atmosphere and showed the interaction between CO_2 and protein in the gas-solid phase system. Among the amino acids that are capable of adsorbing CO_2 are L-lysine and L-arginine, both in free base form. The CO_2 gas adsorption showed, however, some difference with proteins, amines, or amino acids in the moisture dependence and the reversibility.

In this paper, the authors report the results of experiments designed to probe the reactive sites responsible for the CO_2 gas adsorption by proteins. A possible explanation is also presented for the reactivity of a certain particular functional group involved, varying with its existing state, proteins, amino acids and related compounds.

MATERIALS AND METHODS

Materials. The CO_2 gas adsorbing capacity of materials was examined in their solid states. Lysozyme, egg albumin, TNBS, 1,2-cyclohexanedione and other materials used in this experiment were all purchased from commercial sources.

Preparation of TNP-proteins. One gram of protein in concern was dissolved in 50 ml of 4% $NaHCO_3$ (pH 8.5) and incubated at 40°C for 2 hr with the additions of 0 to 100 ml aliquot of 2% TNBS solution. The reaction mixture was dialyzed extensively against distilled water until a pH value lower than 7.0 was reached. The mixture was then freeze-dried. The degree of protein modification was determined by the method of KAKADE *et al.* (*3*).

Preparation of Cbz-Leu-PNP ester. Cbz-Leu-PNP ester was prepared by the method of BODANSZKY *et al.* (*4*). Leucine-PNP ester was prepared from Cbz-Leu-PNP ester by removing of carbobenzoxyl group according to the method of BEN-ISHAI *et al.* (*5*). Identity of both compounds was established by infra-red spectroscopy.

Preparation of protein partial hydrolyzates. Two percent lysozyme solution that had been heated for protein denaturation at 60°C for 15 min was adjusted to pH 9.0 with 0.1 M NH_4OH and was digested with α-chymotrypsine at 37°C for 12 hr. An additional aliquot of α-chymotrypsin solution was added to the reaction mixture after 12 hr and the incubation was continued for another 12 hr. This solution was applied on Sephadex G–25 column to separate large peptides into two fractions. The higher molecular weight fraction was defined as fraction I and the lower one as fraction II, respectively. Fraction I yielded about six times more the fraction II in dry weight basis. Gelatin hydrolyzates were prepared by

the procedure described previously (*2*). Hydrolyzate A′ was prepared by pepsin digestion of gelatin while others were prepared by pepsin and trypsin digestion. All these hydrolyzates were obtained in solid state by freeze-drying.

Carbon dioxide gas. Carbon dioxide gas, which was 99.95% in purity and less than 0.05% in moisture, was commercially obtained and used without any further purification.

Methods. The amount of CO_2 gas adsorbed by the materials was determined by Warburg manometry as described previously (*1*). Well-characterized materials, such as purified proteins and amino acids, allow the describing of the amount of CO_2 gas adsorbed in terms of mole CO_2 per molecule or equivalent of any particular functional groups involved, instead of CO_2 gas volume per unit weight of samples. Some of the previously reported data were reevaluated in such terms and the figures were referred to in this report when they became necessary for comparison. Lysine and arginine contents were determined with a Yanagimoto automatic amino acid analyzer, Model LC–5S, using the SPACKMAN, STEIN, and MOORE procedure (*6*).

RESULTS

Correlation between the amount of CO_2 gas adsorbed and modification degree of egg albumin

Egg albumin samples which differed in the degree of modification by TNBS, *i.e.*, 0, 43, 63 and 100%, were prepared by the procedure described in MATERIALS AND METHODS. The modified sample was ground in a mortar until the whole passed through a 400 mesh sieve, so that the CO_2 gas adsorption data depend least upon the particle size of the samples. In Fig. 1, the amount of CO_2 gas found to be adsorbed by these TNP-proteins are plotted against the percentages of the amino groups remained unmodified in them. It can be seen that the plots give a straight line with the intercept on the ordinate corresponding to CO_2 gas adsorbed by the completely modified protein. It should be noted that the intercept, the CO_2 gas adsorption found with the completely modified protein, gives a measure for nonspecific adsorption of CO_2. Assumed that the intercept value does not vary so significantly with the degree of the modification, the slope of the resultant line can be regarded as representing stoichiometry of CO_2 binding to a functional group susceptible to the TNBS modification, or more precisely the amount of CO_2 per ε-amino group in egg albumin, *i.e.*, 0.087 mole/mole. The amount of ε-amino group in egg albumin was determined as 0.325 mmole/g (14.6 lysine residues per mole protein) by the method of KAKADE *et al.*. This value was almost comparable to the one obtained by amino acid analysis.

The role of amino and guanidinium groups of protein on the CO_2 gas adsorption

Table 1 compares the amount of CO_2 gas adsorbed by hydrolyzates of lysozyme

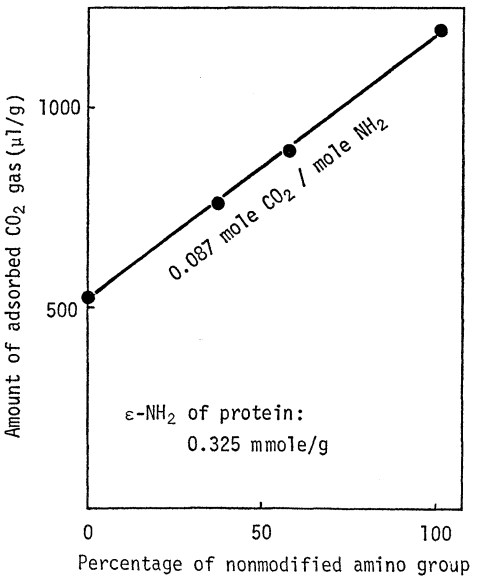

Fig. 1. Correlation between the amount of adsorbed CO_2 gas and modification degree of egg albumin.

Table 1. Amount of carbon dioxide gas adsorbed by protein and protein hydrolyzates.

	Molecular weight	CO_2 gas adsorbed	Lysine content	Arginine content
			(μmoles/g)	
Lysozyme	15,000	6.8	190	464
Lysozyme hydrolyzate				
I	5,000	30.3	424	428
II	<400	14.7	341	355
Gelatin hydrolyzate				
A	700–1,000	39.8	408	541
A′	700–1,000	12.8	257	455
B	1,000–10,000	22.4	200	413
C	>10,000	13.3	253	366

and gelatin, which vary in their lysine and arginine contents and the molecular weight on the average. The higher the degree of hydrolysis, the more the content of free α-amino group is among the series of respective protein hydrolyzate; *i.e.*, the molecular weight of the hydrolyzates is necessarily connected closely with the content of α-amino group. Lysozyme hydrolyzate I and gelatin hydrolyzate B, had molecular weights that were almost the same but total lysine and arginine content that were different; this showed the difference in the capacity for CO_2 adsorption. When the amount of CO_2 was expressed in relative terms to total of

lysine and arginine contents, no significant difference could be detected between those two hydrolyzates in their adsorption capacities. A significant difference is detected in the amount of CO_2 between the gelatin hydrolyzate B and C. This is but mostly attributable to the difference in the amount of free α-amino groups, or conversely in the molecular weight of the hydrolyzates. Correlation coefficients found between the amount of CO_2 and each of the variables, the lysine content, the arginine content and the total of lysine and arginine contents of these protein hydrolyzates were 0.760, 0.539 and 0.847, respectively.

Effect of peptide chain length on the CO_2 gas adsorption capacity of amino groups

Table 2 gives the amounts of CO_2 gas adsorbed by series of oligopeptides and collated with the pK_a value for their α-amino groups reported in the literature (*7*).

Table 2. Effect of peptide chain length on the CO_2 gas adsorption by amino group.

	pK_a values[a] (α-NH_2)	(ε-NH_2)	CO_2/NH_2 (mmoles/mole)
Gly	9.78		0.024
Gly-Gly	8.25		0.111
Gly-Gly-Gly	8.09		0.465
Gly-Gly-Gly-Gly	—		1.47
Polyglycine	7.4[b]		—
Lys	9.18	10.79	3.97
Lys-Gly	—		4.49
Lys-Glu-Ala	—		6.50
γ-Glu-Cys-Gly	8.75		1.03

[a] DAWSON, R. M. C., ELLIOTT, D. C., ELLIOTT, W. H., and JONES, K. M. (Editors), Data for Biochemical Research, Oxford Univ. Press, London (1971).

[b] HAYASHI, K. (*8*).

The rate of CO_2 adsorption, expressed in terms of moles of CO_2 per mole equivalents of amino group, parallels with the peptide chain length, which is in a negative correlation with pK_a values of α-amino group involved. As can also be seen in Table 2, increase in the CO_2/NH_2 values with the chain length differs, however, between the series of glycine peptide and lysine peptide which contain ε-amino group. The increase is significantly higher with the glycine series than with lysine series, although the CO_2/NH_2 values found for the latter are some times higher than those for the former.

The effect of the ionization of carboxyl group on the capacity of α-amino group for CO_2 gas adsorption

Table 3 compares CO_2 gas adsorption capacity of amino acids and their related compounds on a molar basis. The capacity of lysine and arginine to adsorb CO_2 gas is undermined by salt formation through amino and guanidinium groups. Glutamine, asparagine, urea and biuret showed a least capacity of CO_2 gas ad-

Table 3. Amount of carbon dioxide gas adsorbed by amino acids and related compounds.

	CO_2 adsorbed (mmoles/mole)
Lysine	7.94
Lysine monohydrochloride	0
Arginine	1.66
Arginine hydrochloride	0
Arginine glutamate	0.13
Glutamine	0
Asparagine	0
Urea	0
Biuret	0
Histidine	0
Histamine	50.47
Tyrosine	0
Tyramine	3.29
Glutamic acid	0
γ-Aminobutyric acid	0
Leucine	0
Cbz-Leu-PNP ester	1.31
Leu-PNP ester	2.34

sorption. These findings imply that observed larger capacity for CO_2 gas adsorption of lysine and arginine is attributable to the function of ε-amino group and guanidinium group and not to the function of α-amino group nor of amide group. This appears to be applicable also to both acidic and neutral amino acids.

Histamine and tyramine, homologues of histidine and tyrosine, respectively, but lacking in the α-carboxyl group, show a larger capacity in CO_2 adsorption than expected for histidine and tyrosine. γ-Aminobutyric acid, which can be regarded as a homologue of glutamic acid but lacking α-carboxyl group showed least if any capacity for CO_2 gas adsorption.

The CO_2 gas adsorption was clearly discernible with Cbz-Leu-PNP and with Leu-PNP, which are esters with PNP, while unesterified leucine itself showed least capacity. All of these results clearly indicate that α-amino group is the very locus of CO_2 binding in these amino acids and derivatives.

DISCUSSION

In order to probe the particular functional groups involved in the CO_2 gas adsorption by protein, specific chemical modification of ε-amino group of lysine residues was carried out. As is apparent in Fig. 1, about 60% of total amount of CO_2 gas adsorbed by solid egg albumin is attributable to the ε-amino group of lysine residues. This directly provides evidence that the ε-amino group of lysine is capable of adsorbing the CO_2 gas in protein in the same manner as in free amino acid described in the previous paper (*2*). This notion is also supported by the good

correlation between the amount of CO_2 gas and lysine content in protein hydrolyzates (Table 1). As is often the cases with chemisorption, reaction of CO_2 with ε-amino group in the gas-solid phase system does not bear such obvious stoichiometric relation for gas-liquid phase system (*7*). As to the remaining 40% of total amount of CO_2 gas adsorbed by egg albumin, it is not possible at present to attribute it precisely to a particular amino acid residue in protein. The guanidinium group of arginine residues is, however, undoubtedly involved as a functional group.

That α-amino group of protein is involved in the CO_2 gas adsorption is confirmed by sufficient evidence. Although the exact data is not shown in the present paper, the partial hydrolyzates of lysozyme adsorbed more than twice amount of CO_2 gas adsorbed by the intact protein. As can be seen in Table 2, the mole ratio of CO_2 to α-amino group involved appears to increase with the decrease in the pK_a value of the amino group which parallel with its length of the peptide chain. The pK_a values reported for terminal amino groups in tetraalanine and polyglycine are 7.94 and 7.4, respectively (*8*). And, the pK_a value of α-amino group was proven to be lower in protein than in free amino acids, *i.e.*, values of around 7.6–8.0 for the former and around 9.0–10.6 for the latter. Solids and crystals of ampholites, such as amino acids and peptides, would be separated from neutral solutions as dipolar ions rather than as undissociated molecule so that the cohesion state is stabilized by electrostatic forces of attraction between oppositely charged groups. This widely accepted concept does not rule out, however, a possible but not an occasional occurrence of undissociated carboxyl groups and/or unprotonated amino groups. It is also quite feasible to assume that the lower the pK_a of amino group is, the higher the percentage of unprotonated form is in the solid state. As was suggested in the previous paper (*2*), unprotonation of amino group is probably requisite for the adsorption of CO_2 gas. The observed increase in the CO_2/NH_2 values, as shown in Table 2, can thus be best explained in terms of an increased probability of unprotonation of α-amino groups by long range separation of oppositely charged groups possibly due to the elongation of peptide chain.

It has been well established that relative reactivities of a functional group in organic compounds are affected by neighboring groups with various effects, *i.e.*, electrostatic, steric and resonant. For example, Freedman and Radda report the good correlation between pK_a values and nucleophilicity of the amino groups in model compounds (*9*). In amino acids, dipolar ions, the lone electron pair in the nitrogen atom of α-amino group acts as a receptacle for the proton from the α-carboxyl group. The nitrogen atom of this state, ammonium ionic, can not form an additional compound with the CO_2 molecule which is a weak nucleophilic agent. This explains why amino groups exhibit the least capacity for CO_2 adsorption in most neutral and acidic amino acids so far tested in the solid state, while does much in proteins and oligopeptides. This notion is undoubtedly confirmed

by the experimental data shown in Table 3. The CO_2 gas adsorption capacity observed with leucine is substantially nil, but increases markedly on esterification with *p*-nitrophenol of its α-carboxyl group. Carbobenzoxylation of the amino group to yield Cbz-Leu-PNP ester, a secondary amine, appears not to be sufficient for eliminating its adsorption capacity. This implies that the capability of CO_2 gas adsorption by α-amino group of amino acids are hindered by the neighboring carboxyl group.

In conclusion, experimental results obtained in this study are compatible with the conclusion that ε-amino, α-amino and guanidinium groups are the CO_2 binding sites in protein in the gas-solid phase system concerned in this study. Experiments are in progress to explain the reason why the similar functional groups of proteins and amino acids are able to present the different reactivity to CO_2 gas, such temperature dependence, moisture dependence and reversibility, as were shown in the previous paper (*2*).

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