Interaction of N-Benzoyl-(O-Benzoylphenylalanyl-C¹⁴)-Serine with Proteins

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Abstract

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Interaction of N-Benzoyl-(O-Benzoylphenylalanyl-C14)-Serine with Proteins

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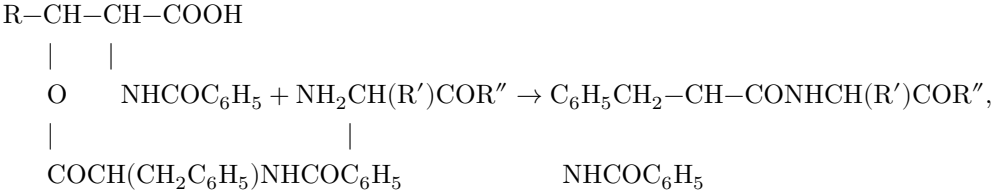
Modern investigations have established that protein synthesis outside the organism can be carried out by various methods ([1]), including transfer of amino-acid and peptide residues from intermediate energy-rich compounds. Thus, in particular, Fruton et al. ([2]) found that synthesis of a peptide chain in a protein can also proceed by transamidation. In the interaction of glycyl-L-tyrosine-C14 amide with insulin in the presence of cathepsin, addition of glycyl-L-tyrosine to the amino group of glycine in chain "A" was observed. Recently we showed ([3]) that O-peptides of serine, threonine, and glycolic acid, in particular N-benzoyl-(O-benzoylphenylalanyl)-serine,* react very readily with esters of amino acids and peptides under the action of chymotrypsin, with formation of esters of optically active peptides



$$\begin{array}{l} \mathrm{R{-}CH{-}CH{-}COOH} \\ \quad | \quad\ | \\ \quad \mathrm{O} \quad \mathrm{NHCOC_6H_5} + \mathrm{NH_2CH(R')COR''} \rightarrow \mathrm{C_6H_5CH_2{-}CH{-}CONHCH(R')COR''}, \\ \quad | \qquad\qquad\quad | \\ \quad \mathrm{COCH(CH_2C_6H_5)NHCOC_6H_5} \qquad\quad \mathrm{NHCOC_6H_5} \end{array}$$

where $\mathrm{R} = \mathrm{H},\ \mathrm{CH_3}$

$\mathrm{R''} = \mathrm{OC_2H_5}$ or the residue of a peptide ester

It seemed of interest to us to test, with the aid of the radioactive-isotope method, the possibility of transferring amino-acid residues from O-peptides of β-hydroxyamino acids to proteins. For this purpose N-benzoyl-(O-benzoylphenylalanyl-C14)-serine was synthesized and introduced into reaction with insulin and blood-serum albumin under conditions previously developed for the enzymatic synthesis of optically active N-peptides from O-peptides of β-hydroxyamino acids. After completion of the reaction, the proteins were precipitated with trichloroacetic acid (TCA), washed with alcohol and

ether, and dried. The radioactivity of the protein was determined with an end-window counter. To remove O-peptide possibly adsorbed on the protein, the preparation was treated with alkali, precipitated with TCA, after which its activity was again determined. Control experiments were set up simultaneously without enzyme or with chymotrypsin previously inactivated by boiling. The degree of incorporation of benzoylphenylalanine-C14 was calculated in μequiv per 1 g of protein per hour and in μequiv per 1 μequiv of protein (Tables 1, 2). The experimental data are summarized in Tables 1, 2, and 3. Comparison of the results obtained with insulin and blood-serum albumin in the presence and absence of chymotrypsin shows that transfer of benzoylphenylalanine-C14 from the O-peptide to the protein is activated by the enzyme. The degree of transfer depends on the molar ratios of O-peptide (donor) and protein (acceptor), and it is different for each of the proteins. Thus, for insulin the degree of transfer increases with increasing molar ratios of donor and acceptor, whereas for blood-serum albumin the transfer is greatest at molar ratios of 5 : 1.

* Hereinafter called O-peptide.

Table 1

Interaction of N-benzoyl-(O-benzoylphenylalanyl-C^{14})-serine with blood-serum albumin

Experiment no.	Molar ratio of components, O—N-peptide: protein	O—N-peptide, mg	Protein, mg	Initial activity per sample, imp/min	Label incorporation, imp/min per 5 mg protein*** after TCA*	Label incorporation, imp/min per 5 mg protein*** after 1 N NaOH**	µeq peptide per 1 g protein per h	µeq peptide per 1 µeq protein****	Enzyme	Note
1	1:1	0.4	60	10800	5*	0**	0	0	—	
2	2.5:1	1	60	27000	173	112	1.8	0.12	Chymotrypsin	
3	2.5:1	1	60	27000	110	62	1.0	0.06	Without enzyme	

Experiment no.	Molar ratio of components, O—N-peptide: protein	O—N-peptide, mg	Protein, mg	Initial activity per sample, imp/min	Label incorporation, imp/min per 5 mg protein*** after TCA*	Label incorporation, imp/min per 5 mg protein*** after 1 N NaOH**	µeq peptide per 1 g protein per h	µeq peptide per 1 µeq protein****	Enzyme	Note
4	5:1	1	27	27000	477	162	2.6	0.168	Chymotrypsin	After 3 h in 1 N NaOH 140 imp/min
5	5:1	1	30	27000	487	173	2.77	0.17	»	After 3 h in 1 N NaOH 140 imp/min
6	5:1	1	30	27000	81	63	1.04	0.06	»	After 3 h in 1 N NaOH 140 imp/min
7	5:1	1	30	27000	160	56	0.90	0.06	»	After 1 h in 1 N NaOH 54 imp/min

Experiment no.	Molar ratio of components, O—N-peptide: protein	O—N-peptide, mg	Protein, mg	Initial activity per sample, imp/min	Label incorporation, imp/min per 5 mg protein after TCA*	Label incorporation, imp/min per 5 mg protein*** after 1 N NaOH**	µeq peptide per 1 g protein per h	µeq peptide per 1 µeq protein****	Enzyme	Note
8	5:1	1	30	27000	57	15	0.25	0.015	—	After 1 h in 1 N NaOH 54 imp/min
9	5:1	1	30	27000	123	26	0.43	0.03	—	After 1 h in 1 N NaOH 54 imp/min
12	0:1	1	13.5	27000	60	23	0.4	0.03	Chymotrypsin	Incubation time 22 h
12	0:1	1	13.5	27000	142	75	1.25	0.08	»	Incubation time 22 h
12	0:1	1	13.5	27000	43	32	0.53	0.035	—	Incubation time 22 h
10	20:1	10	78	270000	17	11	0.19	0.016	Chymotrypsin	
11	20:1	10	78	270000	22	13	0.2	0.016	»	

Experiment no.	Molar ratio of components, O—N-peptide: protein	O—N-peptide, mg	Protein, mg	Initial activity per sample, imp/min	Label incorporation, imp/min per 5 mg protein*** after TCA*	Label incorporation, imp/min per 5 mg protein*** after 1 N NaOH**	µeq peptide per 1 g protein per h	µeq peptide per 1 µeq protein****	Enzyme	Note
13	100:1	20	25	540000	50	4	0.06	0.003	—	Volume of reaction mixture 25 ml
14	100:1	20	25	540000	60	12	0.19	0.012	Chymotrypsin	Volume of reaction mixture 25 ml

* Arithmetic mean of two measurements.
** Arithmetic mean of four measurements.
*** Activity is given with background taken into account.
**** Molecular weight of albumin 65000.

Table 2

Interaction of N-benzoyl-(O-benzoylphenylalanyl-C^{14})-serine with insulin

Experiment no.	Molar ratio of components, O—N-peptide: protein	O—N-peptide, mg	Protein, mg	Initial activity per sample, imp/min	Label incorporation, imp/min per 5 mg protein*** after TCA*	Label incorporation, imp/min per 5 mg protein*** after 0.1 N NaOH**	µeq peptide per 1 g protein per h	µeq peptide per 1 µeq protein****	Enzyme
15	0.65:1	1	20	21975	71	61	1.2	0.007	Chymotrypsin
16	0.65:1	1	20	39222	82	75	0.84	0.005	»
17	0.65:1	1	20	21975	41	11	0.22	0.0012	Without enzyme
18	0.65:1	1	20	39222	91	43	0.48	0.0027	»
19	2:1	3	20	53280	743	168	4.1	0.024	Chymotrypsin
20	2:1	3	20	65925	231	165	3.26	0.02	»
21	2:1	3	20	65925	200	150	3.0	0.018	»
22	2:1	3	20	65925	230	157	3.14	0.019	»
23	2:1	3	20	86310	244	162	2.44	0.014	»
24	2:1	3	20	65925	128	31	0.62	0.0037	Without enzyme
25	2:1	3	20	117666	250	69	0.74	0.044	»
26	2:1	3	20	53280	391	39	0.95	0.006	»
27	2:1	3	20	86310	27	22	0.34	0.0019	PHK
28	2:1	3	20	86310	25	23	0.34	0.0019	»
29	2:1	3	20	86310	46	40	0.59	0.0033	Chymotrypsin inactivated by boiling
30	2:1	3	20	86310	47	37	0.54	0.003	
31	3.3:1	5	20	196110	454	264	2.9	0.017	Chymotrypsin
32	3.3:1	5	20	196110	438	246	2.8	0.016	»
33	3.3:1	5	20	196110	410	55	0.60	0.0035	Without enzyme
34	10:1	15	20	479550	1130	421	5.7	0.033	Chymotrypsin

Experiment no.	Molar ratio of components, O—N-peptide: protein	O—N-peptide, mg	Protein, mg	Initial activity per sample, imp/min	Label incorporation, imp/min per 5 mg protein*** after TCA*	Label incorporation, imp/min per 5 mg protein*** after 0.1 N NaOH**	µeq peptide per 1 g protein per h	µeq peptide per 1 µeq protein****	Enzyme
35	10:1	15	20	479550	1770	61	0.82	0.0047	Without enzyme
36	10:1	30	20	959100	2538	669	9.05	0.052	Chymotrypsin
37	10:1	30	20	959100	2602	73	0.99	0.0057	Without enzyme
38	10:1	30	20	800000	935	796	10.3	0.06	Chymotrypsin

* Arithmetic mean of two measurements.
** Arithmetic mean of four measurements.
*** Activity is given with background taken into account.
**** Molecular weight of insulin is 6000.

To clarify whether the observed radioactivity was due to direct attachment to the protein of N-benzoyl-(O-benzoylphenylalanyl)-serine, and not to enzymatic transfer of benzoylphenylalanine, the serum albumin preparation obtained after incubation and reprecipitation with alkali was treated again with 1 N alkali and left for 1 or 3 hours. The activity was then determined again (Table 1, experiments 5 and 6)—it changed only slightly. Meanwhile, according to the data of M. M. Botvinnik and S. M. Avaeva (3), under these conditions O-peptides are hydrolyzed completely; therefore the protein should have lost its activity. Finally, experiments specially designed for incubation of proteins with benzoylphenylalanine-C^14 showed that the latter is not incorporated into proteins (see Table 3). These negative experiments confirm that incorporation of the label occurs by transfer of the aminoacyl residue from the O-peptide, and not by direct incorporation of benzoylphenylalanine-C^14, which may be formed through hydrolysis of the O-peptide.

Table 3

Interaction of proteins with benzoylphenylalanine-C^14

Experiment No.	Protein	Benzoylphenylalanine, mg	Protein, mg	Initial activity, imp/min	imp/min per 5 mg protein after TCA	imp/min per 5 mg protein after 0.1 N NaOH	Enzyme
39	Serum albumin	0.6	30	16200	3	0	Chymotrypsin
40	Serum albumin	0.6	30	16200	7	4	»
41	Insulin	1.7	20	59755	5	2	»
42	Insulin	1.7	20	59755	14	9	»

The stability of the preparations obtained toward alkali makes it possible to suppose that attachment proceeds through the formation of stable, apparently peptide, bonds. This assumption requires further investigation. Some confirmation of it may be provided by the results obtained by Fruton (2).

Experimental Part

Synthesis of N-benzoyl (O-benzoylphenylalanyl C^14)-serine. Phenylalanine C^14 was obtained from glycine C^14 (in the carboxyl) by the azlactone method (4). M.p. 270–272°; literature data 271–273° (4). Benzoylphenylalanine-C^14 m.p. 184–185°; literature data 186° (5). N-benzoyl (O-benzoylphenylalanyl C^14)-serine m.p. 168°; literature data 171° (6). Yield 21.6%, calculated on the starting glycine. Obtained by the method of M. M. Botvinnik and S. M. Avaeva (6).

Equivalent: found 440 (by titration), 479 (by saponification of the ester bond). $C_{26}H_{24}O_6N_2$. Calculated 460. Specific activity 0.105 mCu per 1 g. Crystalline insulin and crystalline blood-serum albumin were used in the study. Both preparations were electrophoretically homogeneous.

Interaction of the O-peptide with proteins. A weighed portion of protein was dissolved in M/15 phosphate buffer (pH 8.2), and the necessary amount (see Table 1) of O-peptide dissolved in the same buffer was added. The pH of the medium was brought to 8–8.5 by adding 0.1 N NaOH; crystalline chymotrypsin was then introduced (0.2–0.25 mg per experiment). The total volume of the reaction mixture was 2.5–3 ml; it was incubated in a thermostat at 23–24° for 1 hour. The protein was then precipitated with TCA (final concentration 5%). The precipitate was centrifuged, washed 5 times with 5% TCA, 3 times with a mixture of alcohol and ether (1:3), and 2 times with ether, and dried. From 5

mg of the dried incubated protein, a preparation was made on metal plates for determi-

measurement of radioactivity with an end-window counter. The activity was measured with counters of different efficiency, which explains the variation in the specific and initial activity of the O-peptide in different experiments. The magnitude of the standard deviation in measuring the radioactivity of individual samples did not exceed 10%.

Purification of the incubated protein by dissolution in alkali. The protein preparation obtained after incubation was dissolved in 0.1 N NaOH (in the case of blood-serum albumin, in 1 N NaOH) and reprecipitated by adding 10% TCA. The precipitate was centrifuged, washed, dried, and its activity was measured as described above. The results of the experiments are presented in Tables 1, 2, and 3.

Interaction of benzoylphenylalanine-C^{14} with proteins. The experiments were carried out analogously to those described above, except that labeled benzoylphenylalanine was used instead of the O-peptide (see Table 3).

Thus, it has been shown in this work that, under the action of chymotrypsin, N-benzoyl-(O-benzoylphenylalanyl-C^{14})-serine reacts with insulin and blood-serum albumin, with transfer of the benzoylphenylalanine residue to the protein.

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