Chemical approaches to penicillin allergy-IV. Binding of carrier receptor protein with penicillin and its analogues

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MS received 8 December 1977

Abstract. The availability of electrophoretically homogeneous rabbit penicillin carrier receptor protein (CRP) by affinity chromatography afforded an ideal *in vitro* system to calculate the thermodynamic parameters of binding of penicillin and analogues with CRP as well as competitive binding of such analogues with CRP in presence of ^{14}C -penicillin G. The kinetics of association of CRP with 7-deoxy penicillin which does not bind covalently with CRP have been studied through equilibrium dialysis with ^{14}C -7-deoxybenzyl penicillin and found to be $K=2.79 \times 10^4 \text{ M}^{-1}$, $-\Delta G=8.106 \text{ k cal/mole}$ as well as fluorescence quenching studies with exciter $\lambda 280 \text{ K}=3.573 \times 10^4 \text{ M}^{-1}$, $-\Delta G=8.239 \text{ k cal/mole}$. The fluorescence quenching studies have been extended to CRP-benzyl penicillin and CRP-6-aminopenicillanic acid (6APA) systems also. The fluorescence data with benzyl penicillin indicate two conformational changes in CRP—a fast change corresponding to the non-covalent binding to CRP with 7-deoxy penicillin and a slower change due to covalent bond formation. With 6-APA the first change is not observed but the conformational change corresponding to covalent binding is only seen.

Competitive binding studies indicate that the order of binding of CRP with the analogues of penicillin is as follows: methicillin > 6APA > carbenicillin > *o*-nitro-benzyl penicillin > cloxacillin $\approx$ benzyl penicillin $\approx$ 6-phenyl acetamido penicillanyl alcohol $\approx$ 7 phenyl acetamido desacetoxo cephalosporanic acid $\approx$ *p*-amino benzyl penicillin $\approx$ *p*-nitro benzyl penicillin > ticarcillin > *o*-amino benzyl penicillin > amoxycillin > 7-deoxy benzyl penicillin > ampicillin.

From these data it has been possible to delineate partially the topology of the penicillin binding cleft of the CRP as well as some of the functional groups in the cleft responsible for the binding process.

Keywords. Penicillin allergy; hapten binding sites; antigen-antibody reaction; conformation; affinity chromatography; kinetic study and fluorescence quenching.

1. Introduction

In earlier communications of this series the isolation of a specific carrier receptor protein (CRP) and an electrophoretically homogeneous rabbit antipenicillin antibody through affinity chromatography on polymers containing the structural elements of penicillin were reported (Bhattacharyya *et al* 1974, 1975; Nataraj *et al* 1978). It was established that CRP released from the polymeric templates in penicilloylated form by the hydrogenolysis of HF-3 polymer had the strongest affinity of all the serum proteins for penicillin and the conjugate was a full fledged antigen (Bhattacharyya *et al* 1975). The unconjugated penicillin-free rabbit CRP was also isolated by using a polymer with 7-DOHF-3 containing 7-deoxy penicillin as template after elution with 4-8 M urea. Since 7-deoxy penicillin did not have the reactive β -lactam carbonyl

it is incapable of covalently reacting with CRP. It was also shown that CRP itself does not react with the antibody but does so after conjugation with penicillin to form the full fledged antigen (Nataraj *et al* 1978).

The availability of the penicillin-free CRP in pure form and an electrophoretically homogeneous antibody permitted detailed binding studies (a) on the conjugation of CRP with different analogues of penicillin and (b) competitive binding of the conjugates of CRP with penicillin G and other analogues with the antibody sites. Such studies were undertaken with the ultimate objective of developing drugs which would bind with CRP strongly but the conjugate would not bind effectively with the antibody. Such drugs would be safer for administration to patients with penicillin sensitivity. This paper also deals with the kinetics and thermodynamics of binding of penicillin G and other analogues of CRP as well as the competitive binding of ^{14}C -penicillin G in the presence of other analogues.

2. Experimental methods

2.1. Materials

^{14}C -phenyl acetic acid ($-1-c-14$) was obtained from Bhabha Atomic Research Centre, Bombay (each vial is of activity 0.1 mci and specific activity 3.83 mci/m mole). Crystalline potassium penicillin G, 6-amino-penicillanic acid were obtained as gifts from M/s HAL, Pimpri and CIPLA, Bombay. Ampicillin, carbenicillin, ticarcillin, amoxycillin, methicillin and cloxacillin were generous gifts from Beecham Laboratory, UK. The micro-organism used for assay of antibiotic (penicillin G of potency 1640 units/ml) was *B. subtilis* (8236). Infrared spectra were recorded on a Perkin Elmer 700 IR spectrometer and the NMR spectra on a Varian 60T NMR spectrophotometer.

All the UV spectra were recorded using an UNICAM SP 700A double beam automatic recording spectrophotometer.

Perkin Elmer fluorescence spectrophotometer 203 was used for all measurements. Quartz cuvettes of 3 ml capacity and 1 cm light path were used.

2.2. Synthesis of radioactive benzyl penicillin (Chart 1)

^{14}C -phenyl acetic acid was converted to the acid chloride by thionyl chloride by usual procedure. To a stirred solution of 6 aminopenicillanic acid (6 APA) (3 gm) in dry acetone (100 ml), sodium bicarbonate (0.65 gm) in water (3 ml) was added at 0°C . Then ^{14}C -phenyl acetyl chloride in dry acetone (10 ml) was added slowly to the stirred solution maintaining the temperature at 0°C . The stirring was continued for 3.5 hr at 0°C . The mixture was then filtered and the filtrate washed with ice-cold ether. The aqueous layer was diluted with 20 ml ice-cold water and adjusted to pH 6.7 with sodium bicarbonate. Acetone was removed under vacuum below 40°C . ^{14}C -penicillin G(i) was recovered as the crystalline procaine salt after addition of procaine hydrochloride (200 mg). The compound was characterised by TLC, IR, NMR, melting point, potency, specific activity on repeated crystallization. Yield: 8.9 mg. M.P. $125-129^\circ\text{C}$, specific activity on third crystallization $5.930 \times 10^3 \text{ d min}^{-1} \mu\text{g}^{-1}$. Potency 1057 units/mg. IR $\nu_{\text{max}}^{\text{nujol}}$: 3100–3500, 1780, 1700, 1670 cm^{-1} . NMR $\delta(\text{CDCl}_3)$:

8.0 (d, 2H, ArH), 7.5 (s, 5H, ArH), 6.8 (d, 2H, ArH), 5.7 (d, 1H, C₆H), 4.8 (s, 1H, C₅H), 4.6 (m, 4H, NH, BZ CH₂), 4.4 (s, 1H, C₃H), 3.75 (s, 2H, CH₂), 3.2 (m, 6H, (CH₃)₂), 1.6 (d, 6H, C₂(CH₃)₂), 1.3 (t, 6H, CH₃).

Chart 1. Synthesis of radioactive procaine salt of benzyl penicillin.

2.3. Synthesis of *p*-nitro benzyl penicillin (II)

(II) was prepared essentially according to Farbenfabriken Bayer (1962) as detailed in chart 2. The compound was characterised by IR, NMR, TLC, UV and M.P. M.P. 210°C. UV $\lambda_{\max}$: 278 nm in water. (IR $\nu_{\max}^{\text{nujol}}$ 1780 cm⁻¹. NMR $\delta(\text{D}_2\text{O})$: 8.2 d, 2H, ArH); 7.75 (d, 2H, ArH), 5.3 (d, 1H, C₆H), 5.1 (s, 1H, C₅H), 4.1 (s, 1H, C₃H), 3.7 (s, 2H, BZ CH₂), 1.5 (d, 6H, C₂(CH₃)₂).

2.4. Synthesis of *p*-amino benzyl penicillin (chart 2)

The compound (III) was prepared according to the procedure of Tosoni *et al* (1958).

Chart 2. Synthesis of *p*-nitro and *p*-amino benzyl penicillin.

p-amino benzyl penicillin was characterised by TLC, UV, IR, NMR and potency. Potency 1000 units/mg. UV $\lambda_{\max}$, 238 nm ($\log \epsilon$ 4.02) 285 nm ($\log \epsilon$ 3.16). IR $\nu_{\max}^{\text{nujol}}$ 1780, 1700, 3100–3600 cm^{-1} . NMR δ (D_2O): 7.0 (d, 2H, ArH), 6.5 (d, 2H, ArH), 5.6 (d, 1H, C_6H), 5.4 (s, 1H, C_6H), 4.6 (s, 1H, C_3H), 4.4 (d, 2H, BZ CH_2), 1.6 (d, 6H, $\text{C}_2(\text{CH}_3)_2$).

2.5. Synthesis of *o*-nitro benzyl penicillin

The synthesis of *o*-nitro benzyl penicillin (IV) was carried out (chart 3) essentially by the same method described for the synthesis of radioactive benzyl penicillin excepting for the crystallization of final compound which was achieved from a mixture of acetone, water and isopropanol. The compound was characterised by TLC, UV, IR, and NMR. UV $\lambda_{\max}$: 270 nm in water. IR $\nu_{\max}^{\text{nujol}}$ 1780 cm^{-1} . NMR δ (D_2O): 8.1 (d, 1H, ArH), 7.6 (m, 3H, ArH), 5.2 (d, 1H, C_6H), 5.1 (s, 1H, C_5H), 4.1 (s, 1H, C_3H), 3.8 (s, 2H BZ CH_2), 1.5 (d, 6H, $\text{C}_2(\text{CH}_3)_2$).

2.6. Synthesis of *o*-amino benzyl penicillin (V) (Chart 3)

The reduction of (IV) to (V) was also accompanied by essentially the same method as in chart 3 as reported in the case of III. The product was characterised by TLC, IR and NMR. IR $\nu_{\max}^{\text{nujol}}$ 1780, 1680–1660, 3300–3600 cm^{-1} . NMR δ (D_2O): 8.25 (m, 1H, ArH), 7.6 (m, 3H, ArH), 5.2 (d, 1H, C_6H), 5.1 (s, 1H, C_5H), 4.1 (s, 1H, C_3H), 3.8 (s, 2H BZ CH_2), 1.5 (d, 6H, $\text{C}_2(\text{CH}_3)_2$).

Chart 3. Synthesis of (a) *o*-nitro and (b) *o*-amino benzyl penicillin.

2.7. Synthesis of 6-phenyl acetamido penicillanyl alcohol (VI) (Chart 4)

The preparation of the compound (VI) was carried out according to the method as described by Perron *et al* (1964) through the conversion of triethyl ammonium salt of benzyl penicillin to corresponding alcohol with some modification. Triethyl ammonium salt of benzyl penicillin was prepared from the potassium salt of benzyl penicillin by ion exchange chromatography (Amberlite IR 120) as shown in chart 4. The final compound (VI) was characterised by IR and NMR. IR $\nu_{\text{max}}^{\text{nujol}}$: 3280, 1770, 1660, 1530, 1042, 730, 700, cm^{-1} . NMR δ (CDCl_3) 7.28 (s, 5H, ArH), 6.59 (d, 1H, NH), 5.50 (q, 1H, C_6H), 5.27 (d, 1H, C_5H), 3.95 (d, 2H, CH_2), 3.7 (s, 2H, BZ CH_2), 2.24 (d, 1H, OH), 1.40 (d, 6H, $\text{C}_2(3\text{CH}_3)_2$).

Chart 4. Synthesis of 6-phenyl acetamido penicillanyl alcohol.

2.8. Synthesis of 7-phenyl acetamido desacetoxyccephalosporanic acid (VIII) (Chart 5)

The carboxyl protected cepham (VII) (200 mg) was taken in aqueous formic acid (90%, 10 ml). The solution was cooled in ice-water and zinc dust (200 mg) was added and stirred for 1 hr. Zinc was filtered off and washed with aqueous formic acid (2 ml). The filtrate and washings were evaporated in vacuum. Last traces of formic acid were removed by azeotropy with benzene under reduced pressure. The residue was taken in water (10 ml) at pH 3.5 and treated with hydrogen sulphide gas in a warm (40°C) solution for 20 min. The precipitated zinc sulphide was removed by filtration. The aqueous filtrate was lyophilized. The compound was characterised by IR and NMR. IR $\nu_{\text{max}}^{\text{nujol}}$: 3400–3100, 1760, 1670 cm^{-1} . NMR δ (CDCl_3): 7.4 (s, 5H, ArH), 5.9 (d, 1H, C_6H), 5.0 (d, 1H, C_5H), 3.8 (s, 2H, C_2H), 3.2 (d, 2H, BZ CH_2), 2.2 (s, 3H, C_3H).

Chart 5. Synthesis of 7-phenyl acetamido desacetoxy cephalosporanic acid.

3. Bioassay of penicillin

Microbiological method (Collins and Lyne 1976) was used for the determination of potency of procaine salt of ^{14}C -benzyl penicillin G (potency 1640 units/ml based on benzyl penicillin in different concentrations).

4. Radioactivity measurement

The radioactivity of ^{14}C -labelled samples were counted in a Beckmann L5-100 scintillation counter. The counting fluid contained toluene (sulphur-free) and triton X-100 in the ratio 2:1 and 0.5% PPO.

5. Physico-chemical methods

5.1. Stoichiometry of binding of CRP-with radioactive procaine salt of benzyl penicillin

A calculated amount of CRP (160 μg) and benzyl penicillin in the molar ratio (1:20) were incubated for 4.5 hr at 37°C in phosphate buffer saline (PBS) of pH 7.2. The mixture was dialysed at 0°C against 0.05 M PBS for 5 hr by changing the buffer medium after the interval of 1 hr. The dialysis was stopped when the radioactive counts of the buffer medium were almost equal to that of the background count of the scintillation fluid. The CRP-penicillin conjugate inside the dialysis bag was assayed for radioactivity. This experiment was repeated with CRP from different rabbits (both male and female).

5.2. Competitive binding studies of radioactive procaine salt of benzyl penicillin and different analogues of penicillin with CRP

Constant amounts of CRP were mixed with the mixture of radioactive penicillin and penicillin analogues in the following molar ratios 1:4, 4:1 and 1:1 respectively. The conjugates of CRP and benzyl penicillin and penicillin analogues were prepared

by the same procedure as described in section 5.1. After dialyses ^{14}C -penicillin bound to CRP in the presence of analogues were estimated in the scintillation fluid. For each of the analogues a different set of experiments was performed and identical experimental conditions were maintained in each case.

5.3. Determination of association constant of CRP-7-deoxy penicillin

(a) *By equilibrium dialysis:* Since penicillin binds with CRP with a covalent irreversible linkage, the equilibrium dialysis studies were restricted to 7-deoxy penicillin.

Sandwich type dialysis cells made up of perspex were used for this study. Cells were separated by thoroughly washed and dried membrane. CRP was taken on the one side of the membrane while the other side received a solution of ^{14}C -7 deoxy penicillin prepared by the method as described in earlier communication (Nataraj *et al* (1978)) using radioactive phenyl acetic acid. For all the sets, a constant amount of CRP was taken while varying the 7-deoxy penicillin so as to get the respective molar ratios as 1:4, 1:3, 1:2, 1:1, 1:0, 1:0.07, 1:0.05, 1:0.02, 1:0.01. These were equilibrated for 10 days at 0°C . The ^{14}C -7 deoxy penicillin was also placed on the one side of the control cell, whereas on the other side of the control cell only buffer solution of pH 7.2 was employed. The same aliquots of solution were taken out from both the sides at different intervals of time and were assayed for radioactivity. It was found that after eight days the same amount of aliquot from both sides of the control cell gave the same counts indicating that the equilibrium was reached. There was no further change in count for the next two days. The same amount of aliquot was taken from either side of the cell in each experimental set and was assayed for radioactivity.

(b) *Fluorescence quenching:* A solution of CRP ($27\mu\text{g}/2.5\text{ ml}$ of buffer) was taken in dry clean sample cuvette and the same quantity of buffer solution was taken in the reference cell. The pH of the solution was kept at 7.2 and the experiments were carried out at room temperature ($25\text{--}26^\circ\text{C}$). A stock solution of 7-deoxy penicillin containing $3.6\mu\text{g}/\text{ml}$ was prepared. The exciter wave length of fluorimeter was kept fixed at 280 nm and the intensity of the fluorescence emission of the CRP was scanned between 300–360 nm at an interval of 5 nm for both the reference and sample cell with a suitable sensitivity scale. The fluorescence intensities were obtained by subtracting the reference intensity from that of the sample. These emission intensities were plotted against corresponding wavelength to get the fluorescence spectra (figure 6).

The same experiment was performed after each addition of 0.01 ml of 7-deoxy penicillin from the stock solution to both the reference and sample cells. The final readings were taken by adding a large excess of 7-deoxy penicillin (100 times) in a small volume.

5.4. Kinetic studies using fluorescence quenching with benzyl penicillin and 6-amino penicillanic acid (6APA)

The same experiment (5.3) was repeated with both 10 molar and 20 molar excess of benzyl penicillin and with 10 molar excess of 6 APA with a solution of CRP ($27\mu\text{g}/2.5\text{ ml}$) in pH 7.2 buffer.

Table 1. Determination of binding of CRP with radioactive procaine salt of benzyl penicillin.

Rabbit	Amount of CRP (μg)	Activity after binding ($\text{d. min}^{-1} \mu\text{g}^{-1}$)	Bound penicillin (μg)	Mole ratio of CRP: penicillin after binding
M1	160	8974	1.520	0.9502
	160	9750	1.651	1.032
M2	160	10290	1.742	1.089
	160	9375	1.587	0.9920
F1	160	9615	1.628	1.017
	160	10415	1.763	1.102
F2	160	9180	1.554	0.9714
	160	8525	1.443	0.9020

M=Male; F=Female

6. Results and discussion

6.1. Stoichiometry of binding of CRP with penicillin

The results of the determination of the stoichiometry of the binding of CRP and ^{14}C benzyl penicillin are presented in table 1.

It was found that CRP binds with penicillin in 1:1 molar ratio. In case of all the rabbits (both male and female) the same stoichiometry was found to be valid. This also indicates that the CRP and the nature of binding sites of the CRP in all the individual rabbits may be identical.

6.2. Results of competitive binding

In figures 1 and 2 the plots of count ratio against mole fraction of penicillin are presented for various analogues, where the count ratio has been defined as the counts of the

Figure 1. Plot of count ratio vs mole fraction of penicillin. Curve 1. methicillin 2. *o*-nitro benzyl penicillin 3. *o*-amino benzyl penicillin and 4. 7-deoxy benzyl penicillin.

Figure 2. Plot of count ratio vs mole fraction of penicillin. Curve 1, carbenicillin 2, 6-amino penicillanic acid 3, ticarcillin 4, amoxycillin and 5, ampicillin.

CRP-penicillin analogue conjugates divided by the counts given by control (control counts are given by ^{14}C penicillin conjugate only). Mole fraction of radioactive penicillin is expressed as the moles of radioactive penicillin divided by total moles of radioactive penicillin and moles of semisynthetic penicillin. Figures 1 and 2 imply that the analogues represented by the curves running above the line show weaker

Chart 6. Order of binding of penicillin analogues to rabbit CRP.

binding with CRP. The curves with analogues which have almost the same binding strength as penicillin more or less coincide with the heavy line.

The order of the binding energies to CRP is shown in chart 6. From the previous investigation in our laboratory (Bhattacharyya *et al* 1975) it has been demonstrated that benzyl penicillin binds covalently with an exposed amino group of the CRP. In addition to that CRP-penicillin binding involves very specific stereochemical interaction at various sites of the penicillin molecule which is reflected in the specific CRP binding of 7-deoxy penicillin on the affinity template.

In case of the different penicillin analogues with intact β -lactam, except 7-deoxy penicillin which did not have this moiety, a covalent bond is expected to form with CRP and the energy involved due to this covalent bonding would be expected to be of the same order as in that of penicillin G in all cases. But the overall energy of binding would depend on the degree of stereochemical fit of the penicillin analogues in the binding sites of the CRP protein.

The following conclusions can be drawn from the competitive binding experiments.

1. Regarding the thiazolidine and of penicillin (figure 3) moiety, modifications do not materially affect the binding characteristics. For example, 6-phenyl acetamido penicillanyl alcohol in which the carboxyl (COOH) group at position 3 of the benzyl penicillin molecule was modified to alcoholic group (CH₂OH) binds with CRP with the same energy as benzyl penicillin. 7-phenyl acetamido desacetoxyccephalosporanic acid in which hydrophobic site II (figure 3) has been modified, e.g. thiazolidine ring was altered to a six-membered dihydrothiazine system with a methyl group at 3 position and carboxyl group at 4 position also binds to CRP with approximately the same energy as benzyl penicillin. Possibly this thiazolidine portion of the penicillin molecule may be exposed outwards during the formation of the CRP-penicillin complex, a conclusion substantiated by the observation that the head-free polymers with the phenyl end exposed sequesters the CRP more effectively than the tail-free polymer as in Nataraj (1974).

2. Substitution at different positions of the hydrophobic site I (figure 3), on the other hand, results in a considerable variation in the binding strength.

It is interesting to note that the *para* position of the phenyl ring in the hydrophobic site-I does not seem to be involved in the binding with CRP as the binding energies of *p*-nitro, *p*-amino benzyl penicillin and benzyl penicillin with CRP are of the same order. The cleft space of the protein (CRP) (figure 4), around the *para* position is broad enough to accommodate the amino and nitro group. The space may not have any charged group nearby, although the location of an electronegative atom (e.g. an imidazole nitrogen) with hydrogen bonding ability in a specific orientation in the region cannot be ruled out.

Figure 3. Schematic representation of different sites of penicillin.

Figure 4. Cleft space on the protein (CRP).

(Cl) chlorine; (S) sulphur; (N) nitrogen; (O) oxygen and ● hydrogen. — cloxacillin; — benzyl penicillin and - - - methicillin.

Amoxycillin which shows weak binding as compared to benzyl penicillin has a *para* substituted hydroxyl group and a NH_2 group at α position. It shows stronger binding than ampicillin which does not have the *p*-hydroxyl group. This indicates the existence of a group in the cleft, which can form linear hydrogen bonds with the *para* hydroxyl group of amoxycillin making it a better competitor to penicillin than ampicillin. The hydrogen atoms of the *para* amino group in *p*-aminobenzyl penicillin, because of a different bond angle of the N-H group, may not connect with the electro-negative atom on the protein responsible for the hydrogen bonding interaction with a *para* hydroxyl group. This may account for the fact that *p*-amino benzyl penicillin and penicillin have the same affinity for the CRP.

The variation in the α -position in the hydrophobic site-I (figure 3) interfere in the binding strength. For example ampicillin having an amino group in the α -position is the weakest penicillin analogue amongst all those tested for binding with CRP. On the other hand, the affinity for carbenicillin having a carboxyl group in α position is much stronger than ampicillin and superior to that of benzyl penicillin. The difference between these two analogues lies only in the α substitution in which one is positively charged and the other is negative. It is thus logical to conclude that a charge-charge interaction between the CRP and the analogues also play an important role in the binding in the α region of the CRP.

A positively charged amino group (NH_3^+) on the CRP near the α region may repel the $-\text{NH}_3^+$ group of ampicillin while attracting the negatively charged COO^- group at α position in carbenicillin. This charge-charge interaction may add to or subtract from the binding energy of carbenicillin and ampicillin with CRP as compared to that of penicillin. The existence of the positive charge in the cleft space of CRP in the region is also consistent with the strong binding of *o*-nitro benzyl penicillin in which the resonance stabilization of a negative charge on the oxygen at the nitro group is favoured. Consequently the *o*-amino benzyl penicillin is a weak binder.

Among the *ortho* substituted penicillins, methicillin is the strongest binder. In case of methicillin two methoxy groups (OCH_3) are present in both the *ortho* positions and the α -methylene is absent. Although the polar contribution from the methoxy groups to binding to the positive site of the CRP is irrelevant—the entire moiety may make a tighter fit in the α -region of the CRP-cleft by hydrophobic interaction and Van der Waal's forces. Perhaps the methoxy groups overlap with a constriction in the α -region of the CRP slightly beyond the positive site.

That the maximum amount of steric fit is of importance in the binding process may be illustrated by the case of ticarcillin. Since it has an α carboxyl group as carbenicillin, it is expected to show strong binding. However, ticarcillin is somewhat inferior to benzyl penicillin in binding to CRP. This shows that ring size also plays role in the steric fit, as in ticarcillin the phenyl ring is replaced by smaller thiophene ring.

The strong binding of cloxacillin with the CRP (as strong as benzyl penicillin) is rather intriguing. In cloxacillin and methicillin, the orientations of the molecule (figure 4) would indicate that the *o*-chloro group of cloxacillin occupies the same site as one of the methoxy groups of methicillin. The methyl groups on the heterocyclic ring also overlaps with the other methoxy group of methicillin (figure 4). This also gives an idea of the dimension of cleft space in CRP. However, in benzyl penicillin, one of the *meta* positions of the phenyl ring sticks out of the pattern. It may be possible that the CRP cleft may have an attachment point at this region also.

In case of 7-deoxy penicillin, in which the β -lactam at 7 position was reduced synthetically shows weaker binding than benzyl penicillin. It has been established that CRP binds with benzyl penicillin by covalent binding through the opening of the β -lactam ring—with the formation of $(\text{CO}-\text{NH})$ amide bond between the carbonyl group of β -lactam and an exposed nucleophilic unprotonated amino group of CRP in this region. As the probability of forming covalent bond is non-existent with the deoxy analogue, the binding is entirely due to specific stereochemical interaction at various sites of the penicillin molecules with the complementary sites of the CRP. The difference in the energy of binding of 7-deoxy penicillin and penicillin G may be entirely due to the energy of a covalent bond formation. The extra energy is again a difference in energy of a β -lactam and an ordinary amide linkage at 7 position and the CRP-NH_2 .

The question of binding of 6 amino penicillanic acid is somewhat intriguing. From the competitive experiments it appears to show stronger binding than penicillin G for the CRP cleft. This may be due to the fact that the substituent group being absent the compound is free to assume a reverse position in the cleft in such a manner that the carboxyl group of 6 APA comes close to the positive site of the cleft.

6.3. Association constants of $\text{CRP-}^{14}\text{C-7-deoxy penicillin}$ reaction

6.3.1. *By equilibrium dialysis:* The reaction in the present system is given by the following equation:

The apparent equilibrium constant is given by

$$K_{\text{app}} = \frac{x}{(a-x)(b-nx)} \quad (1)$$

a and b are the initial concentration of CRP and ^{14}C -deoxy penicillin respectively. x is the concentration of the CRP ^{14}C -7-deoxy penicillin conjugate and n is the number of binding sites in CRP. The definition of two parameters is as follows:

$$r = \frac{\text{number of moles of penicillin bound to CRP}}{\text{total number of moles of CRP}}$$

$$= \frac{nx}{a}$$

m = concentration of unbound penicillin in moles/l

$$= b - nx.$$

By rearranging the equation

$$\frac{r}{m} = K_{\text{app}} (n - r) \quad (2)$$

$$r = \frac{nx}{a}.$$

This eq. (2) is in the form of Scatchard binding isotherm.

From eq. (2) it is evident that if r/m is plotted against r , K_{app} could be obtained from the slope and n (number of binding sites) could be obtained from the intercept.

Table 2. The results of the equilibrium dialysis experiments on the binding between CRP and ^{14}C -7-deoxy penicillin

No. of sets	r	m (mole/l)	(r/m) (l/mole) ($\times 10^6$)
1	0.1096	4.482×10^{-8}	2.445
2	0.1603	6.515×10^{-8}	2.460
3	0.2607	1.205×10^{-7}	2.154
4	0.2602	1.220×10^{-7}	2.133
5	0.3115	1.559×10^{-7}	1.997
6	0.3590	1.985×10^{-7}	1.809
7	0.4102	2.388×10^{-7}	1.718
8	0.4719	3.040×10^{-7}	1.554
9	0.5219	3.733×10^{-7}	1.398

Figure 5. Scatchard plot for the binding of 7-deoxy penicillin.

From the radioactivity of the solution collected from the compartment which did not contain protein (CRP) in the dialysis cell the concentration of free 7-deoxy penicillin could be determined. From the difference of radioactivity between the two compartments the concentration of 7-deoxy penicillin bound to a known concentration of CRP could be obtained. From these concentrations the values of r , m and r/m for a set of experiments were calculated and presented in table 2. The plot of r/m against r is shown in figure 5 which is a straight line. From the slope of the straight line the apparent association (K_{app}) was found to be $2.79 \times 10^6 \text{ M}^{-1}$. The corresponding free energy has been found to be -8.10 kcal/mole . From the intercept (2.84×10^6) number of binding sites (n) was found to be approximately 1 taking the molecular weight of the CRP to be *ca* 60,000.

The same stoichiometry was also observed for the reaction of CRP with radioactive benzyl penicillin from radioactive assay method.

6.3.2. *By fluorescence quenching:* The association constant of the same reaction was also determined by fluorescence quenching. CRP shows fairly intense fluorescence band with an excitation maximum at 280 nm and a fluorescence maximum at 330 nm shown in figure 6 (curve 1) which corresponds to the tryptophan as the major fluorophore. Figure 6 represents the different fluorescence bands after the addition of different amounts of 7-deoxy penicillin. It can be clearly seen that due to the binding of 7-deoxy penicillin with the CRP the tryptophan fluorescence of the protein is quenched, thus constituting a sensitive indicator of complex formation. 7-deoxy penicillin-CRP titration were carried out at a fixed wave length in order to obtain the association constant of the reaction. Fluorescence intensity has been plotted against volume of 7-deoxy penicillin as shown in figure 7. From the graph the association constant was calculated and found to be $3.513 \times 10^6 \text{ M}^{-1}$ and the corresponding free energy (ΔG) is -8.239 kcal/mole which is very close to that obtained by equilibrium dialysis. K was calculated from the equation:

$$K = \frac{(Fc/TFc)}{(1-Fc/TFc)(n-Fc/TFc)} \times \frac{1}{(\text{CRP})}$$

where F_c = fluorescence quenching after the addition of a definite volume of 7-deoxy penicillin, TFC = Total fluorescence quenching, n = the molar ratio of CRP to 7-deoxy penicillin in the reaction mixture (CRP) = concentration of CRP.

The quantum yield of the CRP was calculated using the following relation in Parker (1968).

$$I_2/I_1 = Q_2A_2/Q_1A_1$$

where I_2 and I_1 are the fluorescence intensities of the solution of protein and standard (tryptophan) respectively, A_1 and A_2 are their respective absorbance and Q_1 and Q_2 are their respective quantum yields.

Using the known value of quantum yield of tryptophan (15%) the quantum yield of CRP at emission maximum (330 nm) when excited at 280 nm was found to be 8%.

6.4. Kinetics of the interaction of CRP with benzyl penicillin

As compared to the fast spectral changes observed during the binding of 7-deoxy penicillin to CRP the overall changes in fluorescence spectra of CRP after the addition of sodium salt of benzyl penicillin were rather slow. It can be seen from figure 8 that the fluorescence spectra of CRP undergoes a sharp and immediate change just after the addition of benzyl penicillin (curves 1 and 2 in figure 8). Another important feature of this change is that the maximum of the fluorescence band has shifted to approximately 337 nm from the original value of 330 nm as in the case of binding of 7-deoxy penicillin with CRP. The decrease in the intensity and the shift of the absorption maximum immediately after the addition indicates that a rapid interaction occurs between CRP and benzyl penicillin before the onset of the slow reaction. This rapid interaction may be the non-covalent interaction between the reacting partners which takes place before the formation of covalent bond and closely resembles the conformation alteration in the binding of 7-deoxy penicillin. The covalent bond formation giving rise to a second alteration in the structure is much slower as

Figure 6. Quenching of fluorescence band of CRP with the addition of 7-deoxy penicillin.

Curve 1. CRP only 2. 0.03 ml deoxy penicillin 3. 0.05 ml deoxy penicillin 4. 0.07 ml deoxy penicillin 5. 0.11 ml deoxy penicillin 6. 0.16 ml deoxy penicillin 7. 0.24 ml deoxy penicillin and 8. 0.40 ml deoxy penicillin.

Figure 7. The titration of 27 µg CRP with 7-deoxy-penicillin at 27°C at pH 7.2 in 2.5 ml of 0.01M phosphate buffer saline solution.

compared to the first change resulting from non-covalent interaction. This is also associated with a small hypsochromic shift of 5 nm indicating second conformational changes, perhaps a partial unfolding of the binding site.

Subsequent changes (slow changes) in the fluorescence spectra (curves 2–7 in figure 8) are characterised by the gradual decrease in the fluorescence intensities with increase in reaction time. There is no systematic change in the emission maxima but the band becomes more and more broad with time. The time dependent change in fluorescence intensity has been exploited for the determination of the rate constant of the covalent binding process or the step II in the sequence.

From the reaction given above, it appears that the reaction would be a second order bimolecular one (first order with respect to benzyl penicillin). But to avoid complication the concentration of benzyl penicillin employed was 10 times higher than that

Figure 8. Quenching of fluorescence band of CRP with time after the addition of benzyl penicillin (10 times).

1. CRP only 2. CRP + benzyl penicillin, 0 min. 3. CRP + benzyl penicillin, 60 min.
4. CRP+benzyl penicillin, 120 min. 5. CRP+benzyl penicillin 200 min.
6. CRP+benzyl penicillin 280 min. and 7. CRP+benzyl penicillin 360 min.

of CRP. In that case the reaction was expected to be pseudo first order and the following rate equation would hold.

$$\ln \frac{a}{a-x} = Kt.$$

Where a is the initial concentration, x is the concentration of the product at time t , and K is the rate constant. Now from fluorescence quenching $a = (I_0 - I_a)$ and $x = I_0 - I_t$ where I_0 , I_t and I_a are the values of fluorescence intensities at the beginning, at a time t and the completion of the reaction respectively. Therefore

$$\log (I_t - I_a) = -\frac{Kt}{2.303} + \log (I_0 - I_a).$$

If $\log (I_t - I_a)$ is plotted against time (t) a straight line should result with a slope $= -K/2.303$ and intercept $= \log (I_0 - I_a)$. The plot is shown in figure 9. From the slope of the straight line the pseudo first order rate constant has been found to be $1.512 \times 10^{-3} \text{ min}^{-1}$.

In order to examine the order of reaction with respect to benzyl penicillin another set of kinetics experiment was carried out with the addition of 20 times excess benzyl penicillin to CRP. The pseudo first order rate constant obtained from the slope of the plot of $\log (I_t - I_a)$ vs time (figure 9) is $2.82 \times 10^{-3} \text{ min}^{-1}$. The pseudo first order rate constant corresponding to 20 times benzyl penicillin is approximately double the value of that corresponding to 10 times benzyl penicillin. This indicates that the reaction is also first order with respect to benzyl penicillin. Hence the actual second order rate constants of the above reaction obtained in two experiments could be obtained by dividing the pseudo first order rate constant by the concentration of benzyl penicillin. The values are $0.8383 \times 10^3 \text{ lit. mol.}^{-1} \text{ min}^{-1}$ and $0.7856 \times 10^3 \text{ lit. mole}^{-1} \text{ min}^{-1}$ which are very close to each other.

Figure 9. First order kinetics plot for the binding of CRP with benzyl penicillin (o 10 times and Δ 20 times) and 6-APA ($\bullet$ 10 times).

From the observed rate constant (K_r) it is possible to calculate the free energy of activation ($\Delta G^\ddagger$) using the following relation $K_r = \left(\frac{KT}{h}\right) \times K^\ddagger$

K_r = rate constant, K = Boltzmann constant and h = Planck's constant.

$$\Delta G^\ddagger = -Rt \ln K^\ddagger$$

From the average value of the second order rate constant $\Delta G^\ddagger$ has been found to be -13.26 kcal/mole.

6.5. Kinetics of the interaction of CRP with 6-APA

Since the strong binding of CRP with 6-APA was an anomalous case the fluorescence quenching studies were conducted with the system. The spectra at different intervals of time are presented in figure 10.

The decrease in intensity just after the addition of 6-APA (10 times) is much less as compared to that of CRP-benzyl penicillin system (curves 1 and 2 in figure 10). Furthermore the absorption spectra do not show appreciable shift immediately after addition. This indicates that primary non-covalent binding of 6-APA with CRP does not produce much change in the conformation of CRP. Subsequently, however, fluorescence intensities decrease with progress of covalent reaction (curves 2–6 in figure 10). The emission maxima shift to higher wave length along with the broadening of the band and the characteristics of this process is very similar to the slower covalent interaction between CRP and benzyl penicillin.

The plot of $\log(I_t - I_a)$ vs time is shown in figure 9 is a straight line indicating that the data fit the pseudo first order rate equation. The pseudo first order rate constant obtained from the slope is $1.943 \times 10^{-3} \text{ min}^{-1}$. The comparison of these two reactions under identical conditions indicates that reaction with 6-APA is faster compared to that with benzyl penicillin. This may be due to the fact that the transition complex of CRP-6-APA has a lower activation energy as it requires less conforma-

Figure 10. Quenching of fluorescence band of CRP with time after the addition of 6-APA (10 times).

1. CRP only 2. CRP+6 APA, 0 min. 3. CRP+6 APA, 60 min. 4. CRP + 6 APA, 120 min. 5. CRP+6 APA, 200 min. and 6. CRP+ 6 APA, 280 min.

tional change in the process. On the other hand, the CRP-benzyl penicillin complex may be characterised with higher activation energy due to the strain produced by larger distortion of the conformation of CRP. This also explains stronger binding of 6-APA as compared to benzyl penicillin, suggesting again that the binding of 6-APA in the CRP cavity (figure 4) takes place by reversing the molecule with no change in fluorescence or conformation. After the covalent binding takes place the cavity may open up to expose the haptenic site for antibody binding as in the case of penicillin G. In fact since the first change in fluorescence spectrum observed in the binding of both 7-deoxy penicillin and penicillin is almost absent, the data with 6-APA only reflect the slow conformational change in the covalent binding process.

Even with the limited number of analogues used in the current work, some clear indication of the topology of the CRP binding site are emerging. Unfortunately the Dreiding models would give only a two-dimensional representation. For a more accurate delineation of the dimension of the cleft space in CRP theoretical quantum mechanical calculations of the conformation of some of the key analogues are necessary. Such studies are already in progress.

Acknowledgement

The authors would like to thank the Council of Scientific and Industrial Research, New Delhi for financial support of the work.

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