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Biochemical evidence for the interaction of regulatory subunit of cAMP-dependent protein kinase with IDA (Inter-DFG-APE) region of catalytic subunit

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Abstract To explore the structural basis required for the holoenzyme formation of cAMP-dependent protein kinase, we have prepared rabbit anti-peptide antibodies that can block the holoenzyme formation without affecting the catalytic activity of the enzyme. The antibodies were raised against a specific site in the catalytic (C)-subunit, termed IDA (Inter-DFG-APE) region, which lies between the kinase subdomains VII and VIII. Although the C-subunit immunoprecipitated with anti-IDA antibodies could not form a stable complex with regulatory (R)-subunit, it was still susceptible to inhibition by the R-subunit or by PKI, a specific inhibitor peptide containing a pseudosubstrate site. These results indicate that there exists an IDA region-mediated interaction between the R- and C-subunits, which is distinct from that mediated through the substrate site and substrate binding site. In accordance with this idea, association of synthetic IDA peptides with the R-subunit was directly demonstrated by resonance mirror analysis. The calculated association constants of IDA peptides were high enough to suggest a possible involvement of the IDA region in the initial step of holoenzyme formation.

Key words: cAMP-dependent protein kinase; Subunit interaction; Resonance mirror analysis

1. Introduction

cAMP-dependent protein kinase (PKA) exists as an inactive holoenzyme composed of a dimer of regulatory (R)-subunit and two catalytic (C)-subunits [1]. It is well established that binding of cAMP to the R-subunit leads to disassembly of the holoenzyme and release of catalytically active C-subunits. Although the molecular mechanism by which the C- and R-subunits constitute the holoenzyme complex remains obscure, it has been shown that a protease-sensitive 'hinge' region in the R-subunit, which contains the substrate consensus sequence of PKA, occupies the substrate binding site of the C-subunit in the holoenzyme [1]. Actually, type II R-subunit can be phosphorylated on the hinge region [2]. However, the substrate site in the R-subunit is unlikely to be the sole region of the interaction, because (1) the highest affinity of known peptide substrates for the C-subunit is in the micromolar range, whereas that of the R-subnit is in the subnanomolar range [3–5], and (2) a mutant R-subunit, in which two arginines in the substrate consensus sequence are replaced by alanines, has been found to form a holoenzyme that can be dissociated by cAMP, although it is no longer a substrate for the C-subunit [6]. These reports suggest the presence of an additional interaction(s) between the R-and C-subunits which is distinct from that through the substrate site of the R-subunit. On the other hand, molecular biological studies on the C-subunit have identified several residues that influence the susceptibility to inhibition by the R-subunit [7,8], and some of them are mapped on the surface of the crystal structure of the C-subunit [9]. However, there is no direct biochemical evidence for the involvement of these residues in the complex formation with the R-subunit.

In the previous study [10], we applied a biochemical approach to investigate the regulatory interactions between the catalytic and regulatory domains of c-Src, a member of the Src family protein-tyrosine kinases. It was found that the kinase activity of c-Src is regulated by an intramolecular interaction between the inhibitory region in the regulatory domain and a region in the catalytic domain, named the IDA (Inter-DFG-APE) region. In the same study, we obtained an antibody against the IDA region that can immunoprecipitate c-Src and make it constitutively active. The IDA region resides in between the conserved sequence motifs in the kinase subdomains VII (DFG) and VIII (APE), and its sequence is highly specific to each protein kinase family [11]. Thus, we proposed that the IDA region might be generally involved in the regulation of protein kinase activity and that the use of anti-IDA antibody will allow us to perform an in vitro activation of certain protein kinases [10]. In the present study, we have utilized synthetic IDA peptides of PKA and anti-IDA antibodies to examine whether the IDA region of PKA is involved in the regulation of PKA activity. The results obtained indicate that the anti-IDA antibodies make PKA constitutively active and that there exists a direct binding between the IDA region of the C-subunit and a site of the R-subunit which is distinct from the substrate site.

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Abbreviations: PKA, cAMP-dependent protein kinase; R-subunit, regulatory subunit; C-subunit, catalytic subunit; PKI, PKA-specific inhibitor peptide; PKC, protein kinase C; IDA region, Inter-DFG-APE region; CTC, carboxyl-terminal peptide of C-subunit; MBP, myelin basic protein; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (35020) was obtained from ICN. cAMP, Kemptide (LRRASLG), and PKA-specific inhibitor peptide (PKI) were the products of Sigma. Sulfosuccinimidobiotin was purchased from Pierce. Protein A-Sepharose was obtained from Pharmacia LKB Biotechnology. Avidin-biotin complex-conjugated alkaline phosphatase kit was from Vector Laboratories. Other reagents of analytical grade were obtained from Wako (Osaka) or Nacalai (Kyoto).

2.2. Peptides and antibodies

The following peptides (aligned in Fig. 1) were synthesized and purified according to the method described previously [10]: IDA1 (FAKRVKGRTWTLCGTPDYIC), IDA2 (FAKRVKGRTWTLC), CTC (CIRVSINEKCGKEFSEF), and IDA-PKC (MCKENMMDGVTTRTFCGTPDYIC). IDA1, IDA2, and CTC correspond to residues 187–205, 187–199, and 335–350 of the bovine PKA C-subunit [12], respectively. IDA-PKC corresponds to residues 484–505 of the rat protein kinase C (PKC) α [13]. The underlined Asp and Ile of IDA1 were introduced in place of the original residues (Glu and Leu, respectively) of the bovine PKA, so that the carboxyl-terminal half of the IDA1 coincides with the sequence of IDA-PKC. The underlined Asn in IDA-PKC is originally His in the rat PKCα and was replaced by Asn of the rat PKCβ [14] for the convenience of peptide synthesis. All peptides except IDA2, which has Cys in its original carboxyl-terminus, were synthesized with an additional Cys in their carboxyl- or amino-terminus for convenience of immunization. A myelin basic protein peptide, MBP_{4-14} (QKRPSQRSKYL), was synthesized as described previously [15]. Rabbit antisera against IDA1, IDA2, and CTC were prepared according to the method of Kishimoto et al. [16], and the IgG fraction was purified as described previously [10]. Biotin-labeled anti-CTC antibody was prepared by incubating the purified IgG (1 mg) with 0.4 mg of sulfosuccinimidobiotin in 0.5 ml of 50 mM sodium phosphate buffer (pH 8.5) at room temperature for 2 h. The coupling reaction was terminated by the addition of 50 μl of 1 M Tris-HCl (pH 7.5), and the labeled antibody was dialyzed against 17.5 mM sodium phosphate buffer (pH 6.3) and used for immunoblot analysis.

2.3. Preparation of C- and R-subunits

The C- and R-subunits of PKA type II were purified from bovine heart according to the methods of Beavo et al. [17] and Dills et al. [18], respectively. Specific activity of the purified C-subunit was 930 nmol/min per mg under our standard assay conditions. One unit of C-subunit activity was defined as the amount of enzyme that catalyzes the transfer of 10 pmol of phosphate from ATP to Kemptide per min at 30°C. A half unit of R-subunit was defined as the amount of R-subunit that inhibits one unit of C-subunit to 50% of the initial activity. The half inhibition concentration of R-subunit for 10.8 nM C-subunit (1.0 unit in 25 μl of the reaction mixture) was 8.8 nM (9.9 ng/25 μl).

2.4. Immunoprecipitation

Immunoprecipitation of the C-subunit was carried out by incubating an aliquot (20 μl) of C-subunit (5 units) with 2 μl of antiserum at 4°C for 2 h. Immune complexes were adsorbed onto 10 μl of protein A-Sepharose, washed twice with IP buffer [1% Triton X-100 and 50 mM Tris-HCl (pH 7.5)], and once with 50 mM Tris-HCl (pH 7.5). The washed immunoprecipitate was then subjected to the kinase assay (see below). Immunoprecipitated C-subunit for immunoblot analysis was prepared by incubating 50 units of C-subunit and 10 μl of antiserum in total 200 μl of sodium phosphate buffer (pH 7.5) at 4°C for 2 h.

2.5. Kinase assay

Kinase assay of the C-subunit was carried out in the standard reaction mixture (25 μl) containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (6.7 Bq/pmol), 100 μM Kemptide, and the immunoprecipitated or nonprecipitated C-subunit. The reaction proceeded at 30°C for 10 min and was stopped by the addition of SDS-sample buffer [19] followed by boiling for 3 min. Phosphorylated Kemptide was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 20% polyacrylamide gel. The gel was dried without staining, and the radioactivity of the specific band was analyzed by a BAS2000 Bioimaging Analyzer (Fujifilm, Tokyo).

2.6. Immunoblot analysis

Immunoprecipitated proteins were separated by SDS-PAGE using 12.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore) by using a semi-dry blotter (Sartorius). The membrane was soaked in T-TBS [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20] containing 1% skim milk for 1 h, and incubated with 10 μg/ml biotin-labeled anti-CTC antibody in T-TBS containing 1% skim milk at room temperature for 2 h. After washing with T-TBS, the membrane was incubated with avidin-biotin complex-conjugated alkaline phosphatase in T-TBS at room temperature for 2 h. To detect the immune complex, 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (50 μg/ml) was used as substrate in the reaction mixture containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$, and 150 μg/ml nitroblue tetrazolium.

2.7. Resonance mirror analysis

Resonance mirror analysis [20] of the interaction between IDA peptides and the R-subunit was performed by using an IAsys cuvette system (Fisons). A cuvette with an aminosilane surface was washed with 10 mM sodium phosphate buffer (pH 7.7), and the surface was activated with 200 μl of 1 mM bis(sulfosuccinimidyl)suberate for 10 min, followed by extensive washing with sodium phosphate buffer. R-subunit (50 μg/ml) in 200 μl of 50 mM sodium acetate buffer (pH 4.0) was immobilized onto the surface at 20°C for 10 min. Immobilized R-subunit gave a signal of 465 arc seconds (465°/3600) after washing the cuvette with the sodium phosphate buffer. The surface was then blocked by incubation with 200 μl of 2 mg/ml bovine serum albumin (Calbiochem) for 5 min. Finally, the cuvette was extensively washed with IAsys buffer [10 mM Hepes-NaOH (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.05% Tween 20]. Association of various IDA peptides onto the surface was measured in the IAsys buffer at 20°C, and the surface was regenerated with 10 mM HCl for 2 min after each binding experiment. Kinetic constants were determined by using the FAST Fit program (Fisons).

3. Results and discussion

3.1. Anti-IDA antibodies inhibit holoenzyme formation without affecting the catalytic activity of C-subunit

To investigate the role of the PKA IDA region lying between kinase subdomains VII and VIII (Fig. 1), we have raised antibodies against this region. Two synthetic PKA IDA peptides, IDA1 and IDA2, were employed as antigens.

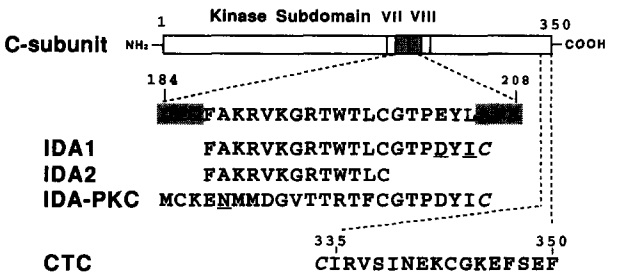

Fig. 1. Schematic structure of the C-subunit and amino acid sequences of synthetic peptides. The structure of the bovine PKA C-subunit is shown with the amino acid sequence of the IDA (Inter-DFG-APE) region lying between kinase subdomains VII (DFG) and VIII (APE). In comparison, the IDA sequence of rat PKCα is also shown. IDA1 and IDA-PKC correspond to the entire IDA region of PKA and PKC, respectively, and share the carboxyl-terminal sequence. The shared sequence is deleted in IDA2. CTC peptide corresponds to the carboxyl-terminal region of C-subunit. Underlined amino acids were substituted and italics represent additional cysteines attached for the convenience of immunization as described in section 2.

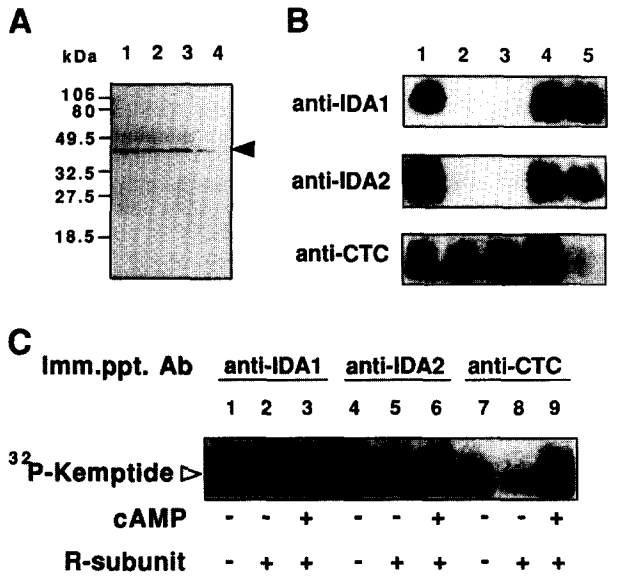

Fig. 2. Anti-IDA antibodies interfere with holoenzyme formation without affecting the C-subunit activity. (A) Immunoblot analysis of immunoprecipitated C-subunit. C-subunit (50 units) was immunoprecipitated with 10 μl of anti-IDA1 (lane 1), anti-IDA2 (lane 2), or anti-CTC (lane 3) antiserum. The immunoprecipitates were subjected to immunoblot analysis with biotin-labeled anti-CTC antibody together with the control C-subunit (2.5 units, lane 4). The position of the C-subunit is indicated by an arrowhead. Prestained molecular size markers (Bio-Rad): phosphorylase *b* (106 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa), and lysozyme (18.5 kDa). (B) Phosphorylation of Kemptide by immunoprecipitated C-subunit. Immunoprecipitation was carried out by incubating C-subunit (5 units) with 2 μl of either anti-IDA1 (top), anti-IDA2 (middle), or anti-CTC (bottom) antiserum which had been preincubated at 4°C for 1 h in the absence (lane 1) or presence of 50 μM synthetic peptides, IDA1 (lane 2), IDA2 (lane 3), IDA-PKC (lane 4), or CTC (lane 5). Immunoprecipitates were subjected to kinase assay as described in section 2. Phosphoimage of ^{32}P-Kemptide was analyzed by BAS2000 Bioimaging Analyzer (Fuji film, Tokyo). (C) Effect of anti-IDA antibodies on holoenzyme formation. Immunoprecipitaion of C-subunit with either anti-IDA1 (lanes 1–3), anti-IDA2 (lanes 4–6), or anti-CTC (lanes 7–9) antiserum was carried out as in (B). The immunoprecipitates were incubated on ice for 10 min in the absence (lanes 1,4,7) or presence (lanes 2,3,5,6,8,9) of R-subunit (1 unit), washed extensively, and subjected to kinase assay in the absence (lanes 1,2,4,5,7,8) or presence (lanes 3,6,9) of 1 μM cAMP.

Since the carboxyl-terminal sequence of the IDA region is highly conserved among PKA and PKC molecules from various species [11], IDA-PKC was prepared to cover the entire IDA region of PKC and used as a control peptide. IDA1 was synthesized so that it coincides with the IDA region of PKC in its carboxyl-terminus. The shared sequence was deleted in IDA2, thus it corresponds to the IDA region specific to PKA. We also prepared a synthetic peptide, named CTC, which corresponds to the carboxyl-terminus of the C-subunit as a control antigen (Fig. 1).

Antibodies against the synthetic IDA peptides (anti-IDA1 and anti-IDA2) and a control antibody against the CTC peptide (anti-CTC) were prepared and characterized. The reactivity and specificity of these anti-peptide antibodies are shown in Fig. 2. Immunoprecipitation and immunoblot analysis revealed that a nearly equal amount of C-subunit was immunoprecipitated with each antibody (Fig. 2A). In Fig. 2B, the kinase activity of these immunoprecipitates was examined by using Kemptide as substrate. It is shown that a similar kinase activity was detected with each immunoprecipitate (lane 1), indicating that the kinase activity of the C-subunit is not affected by the anti-IDA antibodies. Fig. 2B also shows the results of epitope analysis of the antibodies. Anti-IDA1 and anti-IDA2 reacted with both IDA1 (lane 2) and IDA2 (lane 3), but not with IDA-PKC (lane 4) or CTC (lane 5). Reactivity of anti-CTC antibody towards the carboxyl-terminal peptide was also confirmed (Fig. 2B, lower panel).

We next examined whether the C-subunit immunoprecipitated with anti-IDA antibodies is able to form a holoenzyme complex with the R-subunit. This experiment was performed as follows. Immunoprecipitates of the C-subunit prepared as in Fig. 2B were first incubated with an excess amount of the R-subunit. After incubation, immunoprecipitates were washed to remove unbound R-subunit, and then subjected to kinase assay in the presence or absence of cAMP. As shown in Fig. 2C, kinase activity of both anti-IDA1 (lane 2) and anti-IDA2 (lane 5) immunoprecipitates was not inhibited by preincubation with the R-subunit. On the other hand, the kinase activity of anti-CTC immunoprecipitate was clearly inhibited by preincubation with the R-subunit (lane 8), compared with the control activity observed without the R-subunit (lane 7) or in the presence of cAMP (lane 9). These results indicate that the R-subunit cannot form a stable complex with the C-subunit immunoprecipitated with anti-IDA antibodies, while the anti-CTC immunoprecipitate is able to form a holoenzyme.

3.2. Anti-IDA antibodies reduce the susceptibility of the C-subunit to inhibition by the R-subunit, but not by PKI

The finding that anti-IDA antibodies inhibit holoenzyme formation suggested that these antibodies may distort the regulation of the C-subunit by the R-subunit. Thus, we examined the kinase activity of immunoprecipitates in the presence of various amounts of R-subunit. As shown in Fig. 3A, the effect of both anti-IDA1 and anti-IDA2 antibodies was observed as a reduced susceptibility of the C-subunit to inhibition by the R-subunit, whereas the control anti-CTC antibody had virtually no effect. Importantly, however, anti-IDA antibodies

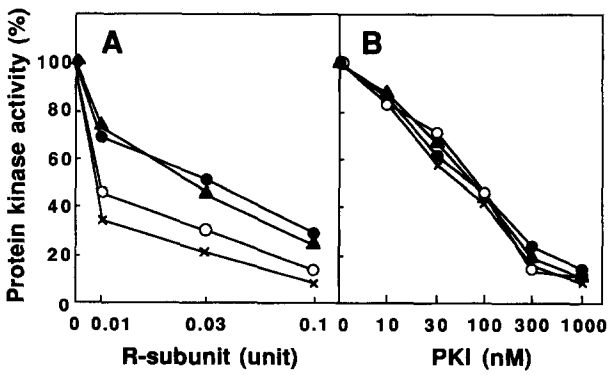

Fig. 3. Effect of R-subunit and PKI on C-subunit activity immunoprecipitated with anti-IDA antibody. Immunoprecipitation of C-subunit with anti-IDA1 (●), anti-IDA2 (▲), or anti-CTC (○) antiserum was carried out as in Fig. 2B. The immunoprecipitated or non-precipitated C-subunit (0.1 units, ×) was subjected to kinase assay in the presence of the indicated amounts of R-subunit (A) or PKI (B). Phosphorylation of Kemptide was quantified as described in section 2. 100% values: anti-IDA1 immunoprecipitate, 4630 PSL; anti-IDA2 immunoprecipitate, 3380 PSL; anti-CTC immunoprecipitate, 4640 PSL; nonprecipitated C-subunit, 4090 PSL.

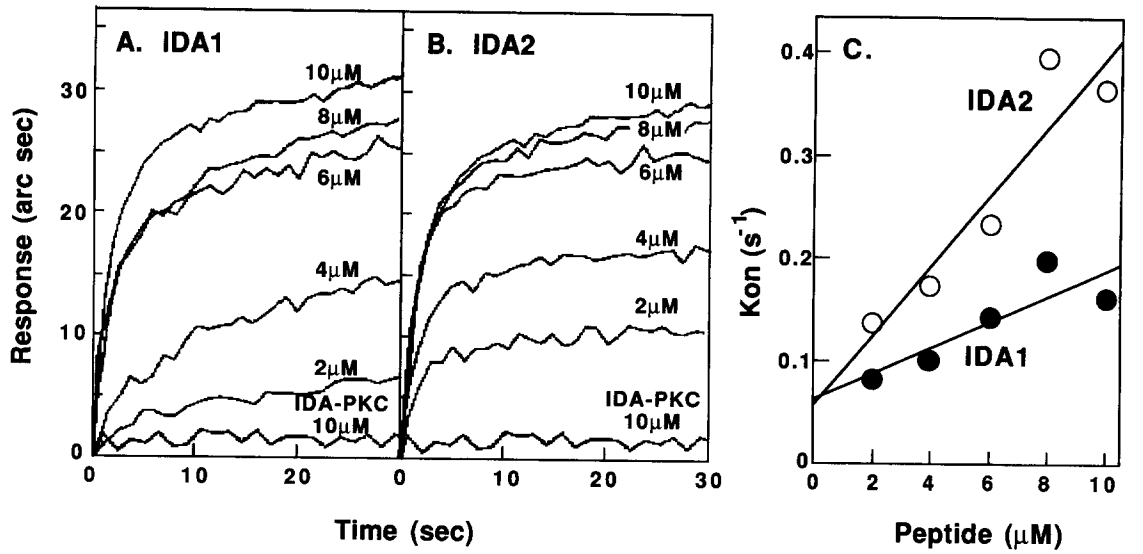

Fig. 4. Resonance mirror analysis of the interaction between IDA peptides and R-subunit. Association of IDA1 (A) or IDA2 (B) with the immobilized R-subunit on the aminosilane surface was analyzed by the IAsys cuvette system as described in section 2. Response is recorded as a change in the resonance angle and represented in arc seconds which is proportional to the amount of peptides associated with the R-subunit. As a control, IDA-PKC (10 μM) was used instead of IDA1 or IDA2. (C) Plots of K_{on} (the on-rate constants for particular concentrations of peptides) against the concentrations of IDA1 (●) and IDA2 (○) were performed by using the FAST fit program. k_{ass} (the slope) and the k_{diss} (the extrapolated intercept on the y-axis) were obtained from the plots. $K_d = k_{diss}/k_{ass}$

could not completely abolish the inhibitory effect of the R-subunit on the C-subunit, indicating that the R-subunit is able to interact with the C-subunit through a site distinct from the IDA region which is blocked by the anti-IDA antibodies. This was confirmed in the experiment using PKI, a PKA-specific inhibitor peptide, which has a pseudosubstrate site similar to the substrate site of the R-subunit [21]. In this case, the concentration-dependent inhibition of the activity of anti-IDA immunoprecipitates followed that of nonprecipitated or anti-CTC-immunoprecipitated C-subunit (Fig. 3B). The result indicates that the pseudosubstrate site of PKI interacts with a site which is different from the IDA region of the C-subunit, and therefore suggests that the interaction site of IDA region in the holoenzyme is different from the substrate site of the R-subunit. The present observation is also quite consistent with the results obtained from molecular biological studies [7,8].

3.3. IDA1 and IDA2 peptides can bind to R-subunit

Further evidence for the participation of the IDA region in the process of holoenzyme formation was obtained by the use of resonance mirror analysis [20]. This enabled direct measurement of IDA peptide binding to the R-subunit. Fig. 4 shows a dose-dependent association of IDA1 (panel A) or IDA2 (panel B) with the R-subunit which was immobilized onto the aminosilane surface. The binding is believed to be specific, because (1) both IDA1 and IDA2 did not bind to the surface on which the C-subunit was immobilized, and (2) a basic peptide MBP_{4-14}, which is derived from myelin basic protein and has a similar pI (pI 11.6) to that of IDA1 (pI 9.5) or IDA2 (pI 11.5), did not bind to the surface on which the R-subunit (pI 4.7) was immobilized (data not shown). Moreover, IDA-PKC, which shares the same sequence with IDA1 in the carboxyl-terminus, did not bind to the immobilized R-subunit (Fig. 4A,B). This observation indicates that the above association is taking place through the IDA2 region.

The stoichiometry of IDA peptide binding to the immobilized R-subunit was calculated from the data for 10 μM and found to be 1.0 mol/mol of R-subunit for IDA1 and 1.3 mol/mol for IDA2. The binding curves, shown as an overlay plot, were analyzed by the FAST Fit program (Fisons) to determine the kinetic constants of binding (Fig. 4C). The association rate constants of IDA1 ($k_{ass} = 1.3\times10^4$ M^{-1} s^{-1}) and IDA2 (3.3×10^4 M^{-1} s^{-1}) were quite high and close to that of holoenzyme formation of the C-subunit with R-subunit type I ($k_{ass} = 1.0\times10^5$ M^{-1} s^{-1}) previously reported by Herberg et al. [5]. In contrast, k_{diss} (IDA1, 6.0×10^{-2} s^{-1}; IDA2, 6.0×10^{-2} s^{-1}) and K_d (IDA1, 4.6×10^{-6} M; IDA2, 1.8×10^{-6} M) were significantly different from those of the holoenzyme formation ($k_{diss} = 2.3\times10^{-5}$ s^{-1} and $K_d = 2.3\times10^{-10}$ M) [5]. The values of the constants indicate that the binding of IDA peptides to the R-subunit is fast but rather unstable.

It is evident that the affinity of IDA peptide to the R-subunit is not sufficient to support a stable complex between the R- and C-subunits. Since the binding of substrate seems to be independent of the IDA-mediated interaction (Fig. 3), it is likely that interactions through both sites (substrate binding site and IDA region) are necessary to achieve efficient binding of the C-subunit to the R-subunit. Considering the high association constant of IDA peptides with the R-subunit, we speculate that the IDA2 region may take part in the initial stage of holoenzyme formation, helping to settle the substrate site of the R-subunit to the substrate binding site of the C-subunit. Identification of the interaction site of IDA2 peptide within the R-subunit will provide further information on this dual mode of interaction between the C- and R-subunits.

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