Volume 333, number 1,2, 96–98 FEBS 13145 October 1993

A novel peptide designed for sensitization of terbium (III) luminescence

Ian D. Clark, Irene Hill, Marianna Sikorska-Walker, John P. MacManus, Arthur G. Szabo*

Institute for Biological Sciences, National Research Council of Canada, Montreal Road, Ottawa, Ont., K1A 0R6, Canada

Received 23 August 1993

Several synthetic peptides, modelled from a Ca^{2+}-binding loop of the EF-hand family of proteins, were prepared containing cysteine residues. The peptide, GDKNADGFICFEEL, was labelled covalently at the cysteine residue (loop position 9) with iodoacetamidosalicylic acid. This novel conjugate is a metal-binding loop containing a salicylic acid side chain that could not only chelate Tb^{3+} in conjunction with the other chelating groups in the sequence, but could also sensitize Tb^{3+} luminescence. The loop had a high Tb^{3+} affinity, with stoichiometric binding observed under experimental conditions. The luminescence from the Tb^{3+}–peptide complex was more than 10-fold greater than the luminescence reported from a related peptide which contained Trp as the Tb^{3+} donor at loop position 7. This peptide has significant potential for use in lanthanide-based time-resolved luminescence immunoassays.

Lanthanide; Luminescence; Peptide; Calcium binding

1. INTRODUCTION

There exists a family of Ca^{2+}-binding proteins that bind the metal ion in a common motif of α-helix, loop, α-helix in the primary sequence. The metal-binding loop portion consists of 12 amino acids that chelate metal ions (Ca^{2+}, Mg^{2+}, Tb^{3+} and Eu^{3+}) through ligand groups at positions 1, 3, 5, 7, 9 and 12 of the loop [1,2]. The carbonyl oxygen of the peptide backbone at position 7 is a ligand, while at positions 1, 3, 5, 9 and 12, chelation involves the residue side chains.

Recently, a consensus sequence Ca^{2+}-binding loop was derived from a database of more than 200 species-variant loops [3] and modified to include different intrinsic chromophores at different non-chelating loop positions [4]. That study showed that the sensitized luminescence of a bound Tb^{3+} ion is particularly high in the 14mer peptide, G(DKNADGWIEFEE)L. The donor chromophore, tryptophan, was at position 7 of the loop, where chelation to the Tb^{3+} is through the backbone carbonyl group. Replacement of the native CD loop of the Ca^{2+}-binding protein, oncomodulin, with this consensus sequence results in a very high level of luminescence from a bound Tb^{3+} ion [5], relative to Tb^{3+} luminescence from a single residue oncomodulin mutant, Y57W, in which the chromophore was placed at position 7 of the existing CD loop. From a series of oncomodulin mutants, the authors found that efficient Tb^{3+} sensitization requires that the donor→Tb^{3+} distance to be <10 Å and therefore suggested that Dexter-type electron exchange is the mechanism of energy transfer [6]. Another oncomodulin mutant was prepared with the consensus CD loop sequence, but containing a cysteine at loop position 7. This allowed the testing of the ability of various sulfhydryl-reactive chromophores to sensitize lanthanide luminescence [7]. This mutant labelled with iodoacetamidosalicylic acid (IASA) was found to be one of the best donors for Tb^{3+} bound in the CD loop. Although salicylic acid conjugated to diethylenetriaminepentaacetic acid (DTPA) is already known to directly chelate Tb^{3+} and sensitize Tb^{3+} luminescence [8], sensitization in the IASA-labelled oncomodulin mutant does not involve direct chelation by the salicylic acid moiety since the CD loop provides all the ligands for the metal ion. Therefore, if a salicylic acid conjugate could be one of the ligands, in conjugation with chelation by the remaining key residues of the binding loop in the protein, an even greater level of Tb^{3+} sensitization could be anticipated due to the effective reduction in the salicylic acid–Tb^{3+} distance.

In this study, 14mer peptides were synthesized containing cysteine at chelating positions 5 and 9 and non-chelating position 7, to allow labelling with IASA and comparison of levels of sensitized luminescence from added Tb^{3+}.

2. MATERIALS AND METHODS

Three 14mer peptides were synthesized by methods previously outlined [4]: GDKNACFIEFEEL (Cys^5), GDKNADGCIEFEEL (Cys^7) and GDKNADGFICFEEL (Cys^9); i.e. cysteine at position 5, 7 and 9 of the binding loop, respectively (note: residues 1 and 14 of the peptide flank the 12-residue binding-loop sequence). Modification of the peptides with iodoacetamidosalicylic acid (IASA) and subsequent purification was as previously reported [7,9]. Solutions of IASA-Cys^5,

*Corresponding author. Fax: (1) (613) 941 4475.

IASA-Cys[7] and IASA-Cys[9] (approximately 8.5 μM) were prepared in 50 mM PIPES, pH 7.0, using ε_{302} = 9,100 $M^{-1} \cdot cm^{-1}$ for IASA. Luminescence titrations were carried out by adding aliquots of a 1 mM stock of $TbCl_3$ solution to 2 ml of peptide. Tb^{3+} luminescence was measured on a SLM 8000C spectrofluorimeter, with data collection by a titration macro. Excitation was at 310 nm, emission was at 545 nm, the temperature was 20°C and all slits were 4 nm.

3. RESULTS

Fig. 1 shows the effect of varying the position of the IASA-labelled cysteine in the 14mer peptide on the amount of Tb^{3+} luminescence obtained. Clearly, the highest Tb^{3+} signal came from Tb^{3+} bound to IASA-Cys[9]. This suggested that, upon replacing the chelating glutamic acid at position 9 with an IASA-labelled cysteine residue, the resulting novel, chelating peptide side chain formed part of the coordination sphere of the bound Tb^{3+} ion. The affinity of IASA-Cys[9] for Tb^{3+} was significant, with stoichiometric binding observed (Fig. 1).

IASA-Cys[7] is the labelled consensus sequence peptide that, in the CD loop of oncomodulin, gave a very high level of Tb^{3+} luminescence [7]. Since chelation at position 7 of the loop is by the backbone carbonyl, the through-space energy transfer from salicylic acid to Tb^{3+} is significantly less than when the salicylic acid is able to chelate the lanthanide directly, as in IASA-Cys[9]. Placement of IASA-labelled cysteine at chelating position 5, in place of the chelating aspartic acid, did not produce the level of Tb^{3+} signal found with IASA-Cys[9]. This suggests that IASA-Cys[5] was not able to improvise a conformation that allowed complete filling of the coordination sphere of Tb^{3+}.

4. DISCUSSION

Recognition of the potential of lanthanide-based, time- resolved luminescence assays as an inexpensive, safe alternative to radioisotope-based assays has grown in recent years. In our laboratory, we have been focusing on the area of protein and peptide-based lanthanide chelation systems [4,5,7,9] which grew from the study of EF-hand Ca^{2+}-binding proteins. In this study, the potential of hybridizing the chelating abilities of a Ca^{2+}-binding peptide and salicylic acid was explored in order to maximize the energy transfer from salicylic acid to Tb^{3+}. Introduction of salicylic acid as a chelating side chain in the peptide was done via labelling of a cysteine group at loop position 5 or 9 with iodoacetamidosalicylic acid (IASA). Salicylic acid was also introduced as a non-chelating donor by labelling of a cysteine at loop position 7. Upon the addition of Tb^{3+}, IASA-Cys[9] clearly gave the largest signal, consistent with the model of Tb^{3+} chelation by both salicylic acid and the peptide. There must be certain conformational constraints present because IASA-Cys[5] did not produce the same level

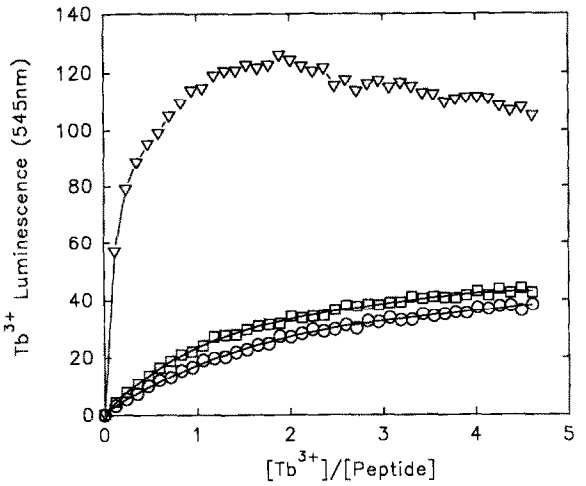

Fig. 1. Tb^{3+} titration of IASA-14mers with cysteine at different positions. Aliquots of a 1 mM Tb^{3+} stock solution were added to 2 ml of IASA-14mer (8.5 μM), with salicylic acid at position 5 (○), 7 (□) and 9 (△) of the loop. Buffer was 50 mM PIPES, pH 7.0. Excitation = 310 nm and emission = 545 nm.

of Tb^{3+} luminescence as IASA-Cys[9], although the same number of potential ligands are present in both peptides. It is known that loop residues 1–5 are more tightly packed than residues 7–12, and this may explain the apparently lower sensitivity of IASA-Cys[5]. The signal from IASA-Cys[7] was also lower than that from IASA-Cys[9] (approximately one-sixth), but this was expected since Tb^{3+} bound in IASA-Cys[7] would have all of its coordination sphere filled by peptide side chains, leaving salicylic acid $\rightarrow$ Tb^{3+} energy transfer to occur through a greater distance in space. The improvement in signal from IASA-Cys[9] over that from peptides previously reported with the chromophore (Trp) at position 7 [4] was greater than 10-fold which reflects not only the importance of the donor–acceptor distance, but the difference between the chromophores' sensitizing abilities.

In conclusion, combining the chelation ability of a Ca^{2+}-binding loop with the chelating and luminescent properties of salicylic acid produced a highly effective method of sensitizing Tb^{3+} luminescence. It remains to be seen if inclusion of IASA-Cys[9] as one of the loops in a Ca^{2+}-binding protein, such as oncomodulin, would produce levels of Tb^{3+} luminescence higher than those currently observed [5,7].

Acknowledgements: We would like to thank Dr. M. Yaguchi, Institute for Biological Sciences, National Research Council, for amino acid analysis of the peptides.

REFERENCES

[1] Kretsinger, R.H. and Nockolds, C.E. (1973) J. Biol. Chem. 248, 3313–3326.
[2] Strydnadka, N.C.J. and James, M.N.G. (1989) Annu. Rev. Biochem. 58, 951–988.
[3] Marsden, B.J., Shaw, G.S. and Sykes, B.D. (1990) Can. J. Chem. 68, 587–601.

[4] MacManus, J.P., Hogue, C.W., Marsden, B.J., Sikorska, M. and Szabo, A.G. (1990) J. Biol. Chem. 265, 10358–10366.
[5] Hogue, C.W.V., MacManus, J.P., Banville, D. and Szabo, A.G. (1992) J. Biol. Chem. 267, 13340–13347.
[6] Dexter, D.L. (1953) J. Chem. Phys. 21, 836.
[7] Clark, I.D., MacManus, J.P., Banville, D. and Szabo, A.G. (1993) Anal. Biochem. 210, 1–6.
[8] Bailey, M.P., Rocks, B.F. and Riley, C. (1984) Analyst 109, 1449–1450.
[9] Clark, I.D., Brown, C.M., Sikorska-Walker, M., MacManus, J.P. and Szabo, A.G. (1993) Anal. Biochem. (in press)