DISUCCINIMIDYL ESTERS AS BIFUNCTIONAL CROSSLINKING REAGENTS FOR PROTEINS

Assays with myosin

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1. Introduction

Bifunctional crosslinking reagents have been used in studies of the spatial arrangement of muscle contractile proteins, such as myosin and actin, either in their soluble forms [1,2], or in synthetic filaments [3–5], or even in myofibrils [3].

The most commonly used reagents are the bis-imidates, which are very reactive, but also quite unstable in aqueous solution; incomplete substitution and unexpected side reactions furthermore occur if the crosslinking reaction is at pH ~8 [6,7]. A disuccinimidyl ester, the dithiobis (succinimidyl propionate), DSP, that does not have these drawbacks and contains, moreover, an easily cleavable disulfide bond, has been described [8]; unlike the bis-imidates, this reagent allowed the crosslinking of the two heads of a myosin molecule [2].

Owing to the high chemical reactivity of DSP and its stability in water, we thought it interesting to synthesize a series of disuccinimidyl esters of various chain lengths (table 1). These include non-cleavable reagents (compounds I–IV), and also reagents with either a *vic*-glycol (compounds V, VI) or an ethylenic bond (compound VII); the crosslinks formed by these last compounds can in principle be cleaved, respectively, by periodate [9,10] and by a mixture of periodate and permanganate [11,12]. Compared to DSP, compounds V–VII present the further advantage of allowing the use during the crosslinking reaction of protein sulfhydryl groups protecting agents.

Evidence of the specific properties of disuccinimidyl esters as bifunctional crosslinkers using skeletal muscle myosin is presented here+.

2. Materials and methods

2.1. *Protein and chemicals*

Myosin from rabbit back and hind-led muscles was prepared by the method in [14] with a further step at ionic strength 0.3 M to remove actomyosin. The reagents used in syntheses were recrystallized. Solvents were distilled and dried on molecular sieves.

2.2. *Synthesis of disuccinimidyl esters*

Disuccinimidyl esters were prepared with a good yield (~70%) using the general method in [15]. The typical procedure for the synthesis of disuccinimidyl succinate I was the following. *N,N'*-dicyclohexylcarbodiimide (4.5,22 mmol) in 20 ml dioxane was slowly added at 15°C to a stirred solution of succinic acid (1.18 g, 10 mmol) and *N*-hydroxysuccinimide (2.52 g, 22 mmol) in 70 ml dioxane. The reaction mixture was allowed to stand at room temperature for 18 h. The precipitated dicyclohexylurea and diester I were filtered, and washed with dioxane; the diester was then extracted with aceto-

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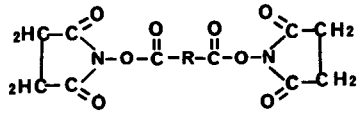
+ While this work was in progress the synthesis of two new disuccinimidyl esters, bridged by a *vic*-glycol bond, one of which identical to compound V, and their use for the study of ubiquinone cytochrome *c* reductase, was reported [13]

Table 1
Analytical and physical properties of disuccinimidyl esters. General formula:



and of one diacid (formula: HOOCR'COOH)

No.	Formula	Length[a]	Recryst. solvent	Mp (°C)		Anal. %			R_F	$\nu^d_{cm^{-1}}$
						C	H	N		
I	$R = (CH_2)_2$	6 Å	Acetonitrile-ethyl alcohol	261	Calc.	46.15	3.85	8.97	0.52[b]	1815, 1780, 1740
					Found	46.01	3.98	9.08		
II	$R = (CH_2)_4$	9 Å	Isopropyl alcohol	168	Calc.	49.41	4.70	8.23	0.60[b]	1810, 1785, 1735
					Found	49.38	4.76	8.07		
III	$R = (CH_2)_6$	11 Å	Isopropyl alcohol	170	Calc.	52.17	5.43	7.60	0.70[b]	1820, 1783, 1725
					Found	52.37	5.42	7.40		
IV	$R = (CH_2)_8$	14 Å	Isopropyl alcohol	164	Calc.	54.54	6.06	7.07	0.74[b]	1815, 1782, 1720
					Found	54.72	6.18	6.90		
V	$R = (CHOH)_2$	6 Å	Isopropyl alcohol	199–200	Calc.	41.86	3.49	8.14	n.d.	1840, 1780, 1720
					Found	41.81	3.43	8.10		
VI	$R = (CHOHCONHCH_2)_2$	13 Å	Ethyl alcohol	145	Calc.	41.92	3.96	12.22	n.d.	1820, 1780, 1730
					Found	41.79	3.91	12.12		
VI'	$R' = (CHOHCONHCH_2)_2$		Ethylacetate-ethyl alcohol	180–181	Calc.	36.36	4.55	10.61	0.87[c]	1641, 1545, 3200
					Found	36.13	4.74	10.53		
VII	$R = (CH_2CH{=}CHCH_2)_2$	9 Å	Ethyl alcohol	175–177	Calc.	49.70	4.14	8.28	0.56[b]	1818, 1780, 1740
					Found	49.85	4.26	8.02		

[a] The length corresponds to the approximate distance between two protein amino groups which the reagent is able to crosslink
[b] Mixture of acetone, methylene chloride and formic acid (100:25:05, v/v/v)
[c] Mixture of ethyl alcohol and water (60:140 v/v)
[d] Imide carbonyl bonds for the diesters; amide carbonyl bond, C–NH and NH bands for the diacid

nitrile, the solution concentrated under vacuum, and the residue recrystallized from acetonitrile–ethyl alcohol.

Minor modifications were used in the syntheses of some of the other diesters. Thus, compounds II–IV, which are soluble in dioxane, were separated from dicyclohexylurea by filtration; the filtrate, evaporated to dryness, left a residue which was recrystallized from isopropyl alcohol. For the synthesis of compound V, the reaction was carried out at –3°C in a mixture of dioxane and ethyl acetate (4:1, v/v). For the synthesis of compound VI, dimethylformamide was used as a reaction solvent at –4°C; the diester, which was soluble in dimethylformamide, was separated from urea by filtration and the filtrate evaporated to dryness, leaving a residue which was isolated by trituration in ethyl alcohol. The diesters V, VI were hygroscopic and were kept in the dessicator.

The diacid VI′ used for the preparation of VI, was synthesized via the *N*-hydroxysuccinimide ester V [15]. A solution of disuccinimidyl tartarate V (2 g, 6 mmol) in dimethylformamide (17 ml) was added to a stirred solution of glycine (0.9 g, 12 mmol) and $NaHCO_3$ (2 g, 24 mmol) in water (30 ml). The reaction mixture was allowed to stand at 4°C for 18 h, then concentrated under vacuum. The residue was triturated in dry ethyl alcohol, then solubilized in the minimum volume of water. The diacid was liberated by addition of HCl (pH 1.5–2.0). After evaporation of water, the residue was dissolved in ethyl alcohol, and the diacid was precipitated by addition of diethyl ether.

The various synthesized compounds were characterized by their infrared spectra (KBr disk), recorded on a Beckman Acculab 2 spectrophotometer, and by thin-layer chromatography on silica-gel plates 60 F (Merck); the spots were detected under an ultraviolet lamp, or in the case of compounds VI, VI′, after developing with 4,4′-tetramethyldiamido-diphenyl methane [16].

The properties of the synthesized compounds are reported in table 1.

2.3. *Crosslinking reactions and reversibility experiments*

Myosin was dialyzed against 0.1 M triethanolamine–HCl buffer, 0.5 M NaCl, 1 mM dithiothreitol (pH 8.5) and used at 2 mg/ml. The crosslinking reagents were dissolved in the minimum volume of dimethylformamide, and added, at final conc. 0.05–3 mg/ml, to the stirred protein solution. The reaction mixture was allowed to stand at 4°C for 2 h. The solution was then made 1% in SDS and heated for 5 min in a boiling water bath; it was finally dialyzed against 0.02 M phosphate buffer (pH 7.0), 1% SDS and analyzed by SDS–gel electrophoresis.

Reversibility of myosin crosslinking with compounds V, VI was achieved by oxidation in the dark with sodium periodate. Experiments were performed both in solution and in the slice of gel in situ. In the first case, the crosslinked sample was incubated for 90 min with periodate (molar ratio, periodate : crosslinker 0.5:2) in the SDS dialysis buffer. In the second case, slices of gels corresponding to crosslinked myosin were cut out, either after a rapid staining, or by careful alignment with a stained gel; they were then incubated for 3 h in the SDS dialysis buffer containing $NaIO_4$ (15 mM). When compound VII was used as the crosslinker, the oxidation was carried out in solution for 15–120 min with a mixture of 0.1 M periodate and 2 mM permanganate. In all cases, controls with non-crosslinked species were made.

2.4. *Sodium dodecyl sulphate electrophoresis*

SDS–electrophoresis was performed according to [17]. Acrylamide (3%) cylindrical gels (60 × 5 mm) were used and run for 2.5 h at 8 mA/tube. Analysis of the slices of gel which were cut out after the first run and treated by periodate was achieved by a second electrophoresis on 4% acrylamide gels (60 × 6 mm) for 6.5 h at 8 mA/tube. The gels stained with Coomassie brillant blue were scanned with a Vernon PHI 6 densitometer equipped with an integrator.

3. Results and discussion

All the compounds synthesized allowed the intramolecular crosslinking between the two heavy chains HC of myosin; an example of the formation of the dimer HC_2 is given in fig.1, in the case of *N,N′*-bis (succinimidylacetate) tartaramide VI. For maximal production of dimer without concomitant formation of any appreciable amount of intermolecular *n*-mers, non-cleavable disuccinimidyl esters (I–IV) and com-

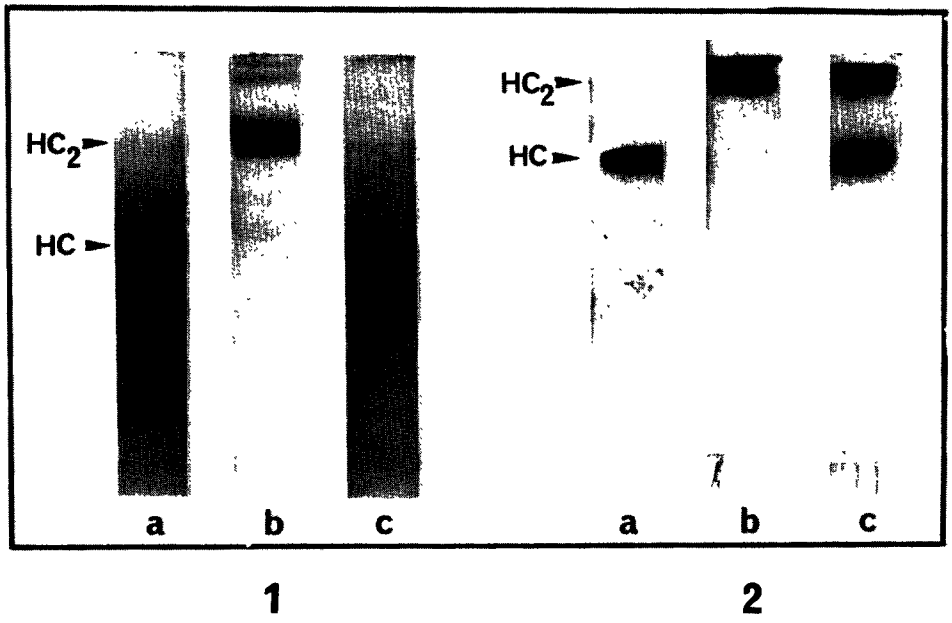


Fig.1. Crosslinking of myosin by *N,N'*-bis (succinimidylacetate) tartaramide VI and its reversal by periodate. Electrophoresis in the presence of SDS : HC represents the 200 000 mol. wt heavy chain and HC_2 the crosslinked dimer. 1. (a) untreated control myosin; (b) crosslinked myosin; (c) crosslinked myosin treated by periodate. 2. Re-electrophoresis of single zones cut from 3% acrylamide gels (as in 1) and run in 4% gels: (a) untreated heavy chain HC; (b) crosslinked heavy-chain dimer HC_2. (c) HC_2 after incubation with periodate, showing regeneration of ~50% of the monomers.

pound VII were used at a weight ratio R/P of the reagent R to the protein P of the order of 0.1; in the case of the tartaric acid derivatives (V, VI), R/P was found $\simeq$0.75 (fig.2).

Reversibility, achieved by periodate oxidation of the crosslinks introduced by these last two compounds (V, VI), was total in solution and about half complete in the gel in situ; in our experimental conditions, it was moreover not accompanied by any substantial damage to the protein (fig.1). On the other hand, the mixture of periodate and permanganate, used for the cleavage of crosslinks introduced by disuccinimidyl β-hydromuconate VII, induced an important degradation of myosin.

Crosslinking of both heads of a single myosin molecule, as described with DSP [2], was also obtained, this time in the presence of 2-mercaptoethanol, with the non-cleavable analogous disuccinimidyl ester III. No disulfide exchanges could have therefore been responsible for the reaction which shows that the heads in intact myosin are at least partly in contact.

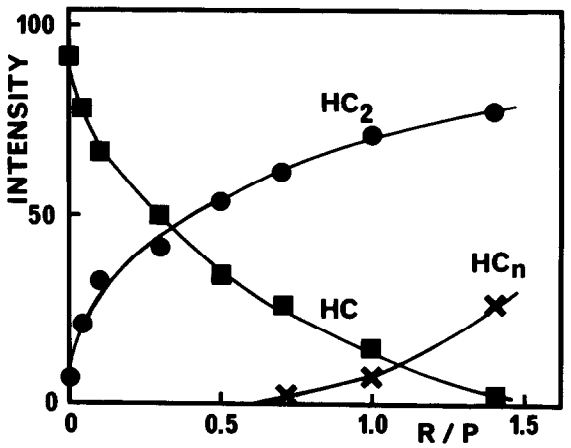


Fig.2. Variation of the relative proportions of myosin monomers HC (▪), dimers HC_2 (•) and n-mers HC_n (×) with the weight ratio R/P of the crosslinker VI to the protein. The protein concentration was constant at 2 mg/ml. Intensities (in arbitrary units) of the bands corresponding to each myosin species were determined by quantitative scanning of the gels.

Disuccinimidyl esters as crosslinking reagents present a number of advantages. Their synthesis, from commercial carboxylic diacids, occurs in one step with good yields; they are relatively stable in aqueous solution and at the same time very reactive. The parallel use of reagents containing either a disulfide bridge, or a *vic*-glycol bond, as well as of the non-cleavable control analogs should prove fruitful in further studies of the spatial organisation of myofibrils.

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