ACRYLAMIDE FLUORESCENCE QUENCHING STUDIES ON SKELETAL AND CARDIAC TROPONINS

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1. Introduction

Fluorescence quenching is an excellent method to determine the degree of exposure of tryptophan residues in proteins [1]. Small molecules had been used as fluorescence quenching agents, e.g., I^-, NO_3^-, Cs^+ and molecular oxygen, but these reagents were not always well-suited to estimating the degree of exposure of aromatic chromophores. To avoid the problems of ionic, hydrated quenchers and the diffusibility of molecular oxygen, the neutral quenching probe, acrylamide, has become popular, since it is discriminatory in its ability to sense degrees of chromophore exposure [2,3].

This work studies the acrylamide quenching of the tryptophan-containing subunits TN-I and TN-T of the skeletal and cardiac troponin complex, and some representative inter-protein complexes prepared in vitro from the individual members. Reconstituted troponin is also compared with the native material isolated without the use of denaturants. The results indicate partial burying of the tryptophan(s) both in TN-I and TN-T. The cardiac analogs seem to have less accessible chromophores than the skeletal analogs. Upon complex formation with TN-C, further burying was noted in the skeletal system, which was not so apparent with the cardiac proteins. Native undissociated troponin has less accessible tryptophans than reconstituted troponin. These results are discussed briefly in the light of known protein sequences.

Abbreviations: MOPS, morpholinopropanesulfonic acid; TN, troponin; TN-C, troponin C; TN-I, troponin I; TN-T, troponin T; s and c refer to the proteins from skeletal and cardiac muscle, respectively; *N*-Ac-Tryp-OEt, *N*-acetyl-tryptophan-ethyl ester; Pipes, piperazine-*N*,*N*'-bis[2-ethanesulfonic acid]

2. Materials and methods

2.1. *Protein preparations*

Crude troponin was isolated from minced bovine heart muscle, or rabbit skeletal muscle by the LiCl extraction procedure [4], as modified [5]. Native troponin was purified by ion-exchange chromatography on DEAE-Sephacel as in [6]. The troponin subunits were purified by ion-exchange chromatography under denaturing conditions in 8 M urea solutions [7–9]. Protein concentrations were determined spectrophotometrically using $E^{1\ mg/ml}_{276\ nm} = 0.43$ for native troponin, 0.23 and 0.19 for cTN-C and sTN-C, 0.37 for TN-I and 0.39 for TN-T. M_r values of the subunits were taken as 18 000 (sTN-C), 18 500 (cTN-C), 20 500 (sTN-I), 22 900 (cTN-I), 30 500 (sTN-T) and 36 300 (cTN-T). Complexes of TN-I with TN-C were prepared by dissolving approximately equimolar amounts of each subunit in 6 M urea, 25 mM Tris–HCl (pH 7.5), 1 mM DTT, 1 mM $CaCl_2$, combining them, and passing the solution through a column of DEAE–Sephadex A-25 equilibrated with the same solvent. The TN-IC complex is stable under these conditions and may be isolated by application of a salt gradient to 0.5 M NaCl. Urea and salts were eliminated by dialysis against water, and the protein obtained by lyophilization. Equimolar amounts of the other subunit complexes were mixed in benign medium and the fluorescence measured.

2.2. *Fluorescence measurements*

Fluorescence measurements were done on a Perkin-Elmer MPF-44B spectrofluorometer operating in the ratio mode with 5 nm bandwidths for excitation and emission slits. Measurements were made at 20°C. A constant temperature was maintained in the cell by

circulating water through the cell holder from a Lauda K2R apparatus. The excitation wavelength used was 295 nm (to ensure that the light was absorbed almost entirely by tryptophan residues). In all instances the initial A_{295} of the protein solution was ≤0.05; thus avoidance of the inner-filter effect was assured. The fluorescence quenching was measured at the emission maximum of the protein and was initiated by addition of 10 μl aliquots of 8 M acrylamide solutions. Cells of 1 cm pathlength were employed and stirring was by a magnetic 'flea'. Protein solutions of 2 ml were used. The F values were corrected for the acrylamide absorption using:

$$F_{corr} = F \cdot 10^{A/2}$$

where A is the absorbance in the 1 cm cell at 295 nm.

2.3. *Data handling*

Fluorescence quenching data is most often described in terms of the Stern-Volmer equation:

$$\frac{F_o}{F} = 1 + K_{SV}\,[Q] = \frac{\tau_o}{\tau} \qquad (1)$$

where τ_o and τ are the fluorescence lifetimes in the absence and presence of quencher (Q), and F_o and F represent the fluorescence intensities at the emission maximum, in the absence and presence of Q. The collisional quenching constant, K_{SV}, may be obtained from the slope of a plot of F_o/F vs [Q]. For the specialized case of a protein with only a single tryptophan, the Stern-Volmer equation has been modified to adequately define the overall quenching process [2]:

$$\frac{F_o}{F} = (1 + K_{SV}\,[Q])\, e^{V[Q]} \qquad (2)$$

$$\frac{F_o}{F_e^{V[Q]}} = 1 + K_{SV}\,[Q] \qquad (3)$$

In these equations, the kinetics of the quenching reaction are separated into a collisional (defined by K_{SV}) and a static (defined by V) component. In practice, one plots $F_o/F_e V[Q]$ vs [Q] for different values of V until the data is linearized.

In [1] the Stern-Volmer equation is modified as:

$$\frac{F_o}{F - F_o} = \frac{1}{f_a} + \frac{1}{f_a K_q [Q]} \qquad (4)$$

where f_a is the fraction of the fluorescence to which the quencher [Q] has access, and where each accessible chromophore has the same Stern-Volmer quenching constant K_q. A plot of $F_o/F - F_o$ vs $[Q]^{-1}$ in this special case will be linear with f_a and K_q being obtained from the slope and intercept, respectively. The data in tables 1,2 was derived from both types of analysis.

3. Results and discussion

3.1. *Single tryptophan proteins*

The results of typical acrylamide quenching experiments for sTN-I and cTN-I are shown in fig.1. The Stern-Volmer plots in both cases reveal upward curvature, typical of single-tryptophan proteins which show a significant degree of quenching [2]. The data can be linearized to separate the quenching into collisional and static components via eq. (3) (also in fig.1). These quenching experiments have been repeated in several solvent systems. Protein complexes with TN-C have also been studied. The full list of quenching parameters is displayed in table 1. Also included are parameters for the model compound *N*-Ac-Tryp-OEt which is considered to be representative of a fully exposed tryptophan. The K_{SV} values for sTN-I and cTN-I are significantly different; 8.16 M^{-1} and 6.58 M^{-1} suggesting that the tryptophan in sTN-I is more exposed than in its cardiac counterpart. The V values are inherently less accurate (±25%) and hence cannot be differentiated. The value of 0.6 suggests some degree of burying of the tryptophan compared with 1.5 for *N*-Ac-Tryp-OEt fully exposed, and 1.0 for the

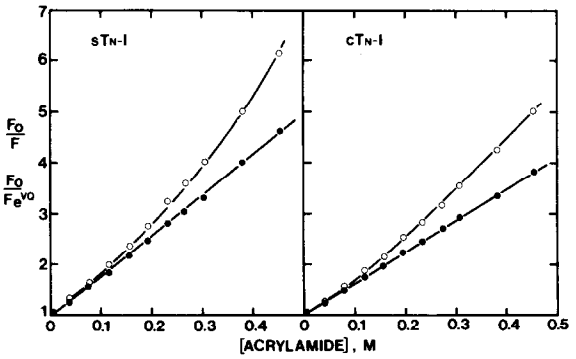

Fig.1. Acrylamide quenching of fluorescence for the single tryptophan-containing proteins skeletal and cardiac TN-Is. The separation into the static and collisional components follows eq. (3). (○) F_o/F; (●) $F_o/F_e V[Q]$.

Table 1
Acrylamide quenching parameters for single tryptophan containing subunit TN-I

Protein	K_{SV} (M^{-1})	V (M^{-1})	$k_q \times 10^{-9}$ [a] ($M^{-1} \cdot s^{-1}$)	K_q (M^{-1})	λ_{max} (nm)
N-Ac-Tryp-OEt	10.6	1.5	4.09	10.5	348
sTN-I[b]	8.16	0.6	3.14	7.42	343
sTN-I[d]	8.30	0.7	3.19	7.21	345
sTN–IC[e]	6.49	0.5	2.50	7.15	340
cTN-I[b]	6.58	0.6	2.53	7.65	341
cTN-I[c]	6.40	0.6	2.46	7.09	340
cTN-I[d]	7.20	0.75	2.77	7.21	345
cTN–IC[e]	6.48	0.3	2.49	6.95	340

[a] An invariant lifetime of 2.6 ns is assumed in these estimations of k_q
[b] 0.3 M NaCl, 10 mM MOPS, 2 mM EDTA ± 2 mM free Ca^{2+} ± 1 mM DTT, (pH 7.2)
[c] 0.015 M NaCl, 10 mM Pipes, 2 mM EDTA (pH 6.8)
[d] 8 M urea, 2 mM EDTA, 10 mM MOPS (pH 7.2)
[e] 0.3 M NaCl, 25 mM MOPS, 1 mM $CaCl_2$ (pH 7.2)

protein ACTH, largely exposed [2]. These results accord with [10] where a detergent-induced difference spectrum of this protein had suggested that the tryptophan chromophore was ~50% exposed. In comparing the quenching constants for the two proteins it has been assumed that the τ_0 values are equal and since we have not been able to actually measure these lifetimes, 2.6 ns has been used throughout.

The values of K_{SV} and V for sTN-I obtained in benign medium and 8 M urea are almost unchanged. Circular dichroism (CD) studies showed that the protein lost ~86% of its original ellipticity when transferred to 8 M urea, suggesting extensive unfolding of the molecule (unpublished). It is known from sequence studies that tryptophan is located at position 158 for skeletal TN-I and 189 for rabbit cardiac TN-I, in a region of the polypeptide chain predicted to be in the random configuration [11] and thus will be little affected by the unfolding of the rest of the molecule.

Comparing K_{SV} = 8.16 M^{-1} for sTN-I and K_{SV} = 6.40 M^{-1}, cTN-I, the tryptophan in sTN-I appears slightly more exposed than the tryptophan in cTN-I. This is suggested also by the values of the fluorescence emission maxima for the two proteins: 343 nm for sTN-I and 340 nm for cTN-I, compared with 345 nm for both proteins in urea solution.

TN–IC complexes: When sTN-I interacts with sTN-C to produce TN–IC there is a significant blue shift in the fluorescence emission maximum, 343 → 340 nm. The K_{SV} and V terms are also significantly reduced, implying that the tryptophan is becoming more buried. For cTN–IC the blue shift is noted along with a considerable reduction in the V term. However the K_{SV} value is essentially unchanged from that obtained for cTN-I alone. These data suggest sensitive differences in the way TN-I from the two sources interact with their respective TN-Cs.

In both sTN-I and cTN-I the TN-C binding region is located near the N-terminus [11,12] which is considerably removed from the tryptophan location. However the result of secondary and tertiary folding of the polypeptide chain could have the effect of bringing the tryptophan close to the TN-C binding domain, where its environment would be perturbed by the interaction with this protein. This effect is apparently more pronounced with the skeletal proteins.

3.2. *Multi-tryptophan proteins*

The fluorescence quenching parameters for sTN-T, cTN-T and representative complexes involving these two subunits are listed in table 2. Also included is data on native troponin from the two sources. A consideration of the quenching parameters suggests that the tryptophans in TN-T are only partly exposed, with those in the cardiac analog being more buried, again in agreement with detergent perturbation results [10]. Emission maxima for the proteins are different, 343 and 340 nm for sTN-T and cTN-T, respectively. Urea unfolding affects these quenching parameters signifi-

cantly, particularly so with cTN-T. Sequence studies on sTN-T have located the positions of the two tryptophan residues. One is the penultimate residue to the C-terminus, while the other is located at position 205. The residue near the C-terminus is predicted to be in an aperiodic region, while that at position 205 is predicted to be at the beginning of a stretch of β-sheet [13], and would be significantly affected by urea treatment. Unfortunately, as yet the sequence of cTN-T is not known so no comments can be made on the location of the tryptophan residues.

When sTN–CT is formed there is a 4 nm blue shift in the emission maximum compared to sTN-T and a considerable reduction in the quenching parameters suggesting a significant additional burying of the tryptophan chromophore upon complexation. With cTN–CT, the blue shift is smaller, ~2 nm, and the quenching constants are little altered from those of cTN-T alone, again indicative of differences in the way the proteins from the two different tissues interact with each other.

The sTN–IT complex has been included, although since both partners contribute tryptophan residues the quenching results are difficult to interpret. From the blue shift of the emission maximum and the reduction in the quenching parameters, it would appear that relative to sTN-I and sTN-T, some additional burying of the chromophore(s) is induced. This is in accord with CD and absorption difference spectral results which indicated perturbations in the environment of aromatic residues upon complex formation and their transfer to a more hydrophobic environment [14].

From recent sequence studies on sTN-T [15], the regions of the polypeptide chain responsible for interaction with TN-I, TN-C and tropomyosin have been elucidated. The interaction site for TN-I appears to be 2-fold; residues 1–70, the N-terminal region and 152–209. The TN-C binding site is from 159–259, the C-terminal region. The binding site for tropomyosin lies in the region 71–151. The two tryptophan residues in sTN-T are located in the TN-C binding site (tryptophan 205 also finds itself at the tail end of the second proposed TN-I binding site). Hence it is not surprising to find that the environment of these groups is significantly perturbed, particularly on formation of the TN–CT complex and to a lesser extent the TN–IT complex. Although the sequence of cTN-T is not known it appears from the fluorescence quenching results that the mode of interaction with cTN-C is somewhat different than sTN-T.

We have compared the fluorescence quenching parameters of native troponin with those of reconstituted material prepared by combination of separately purified subunits. The quenching parameters and the wavelength of the emission maximum for native troponin are all lower than the values obtained for the reconstituted protein, suggesting that the chromophores are considerably more buried in the native protein. When the skeletal proteins are unfolded in 8 M urea there is much closer agreement between the various quenching parameters and the values indicate a high degree of chromophore exposure. For the car-

Table 2
Acrylamide quenching parameters for the multi-tryptophan containing troponin subunits

Protein	$K_{SV(eff)}$ (M^{-1})	$k_{q(eff)} \times 10^{-9}$ [a] ($M^{-1} \cdot s^{-1}$)	K_q (M^{-1})	λ_{max} (nm)
sTN-T[b]	8.02	3.09	6.60	343
sTN-T[c]	9.72	3.74	8.27	347
sTN–CT[d]	5.97	2.30	5.08	339
sTN–CT[e]	6.40	2.46	5.71	339
sTN–IT[f]	7.34	2.82	6.30	341
sTN–ICT[g]	7.28	2.80	6.19	341
sTN–ICT[c]	9.63	3.71	7.87	345
sTN[g]	5.55	2.13	4.95	340
sTN[c]	9.10	3.50	8.06	347
cTN-T[b]	6.8	2.62	5.95	340
cTN-T[c]	10.0	3.85	8.76	348
cTN–CT[d]	6.43	2.47	6.35	338
cTN-T–sTN-C[d]	5.72	2.20	5.71	338
cTN–IT[f]	6.87	2.64	5.97	340
cTN–ICT[g]	8.05	3.09	7.06	341
cTN–ICT[c]	9.49	3.65	8.86	348
cTN[g]	5.13	1.97	5.44	340
cTN[h]	5.33	2.05	6.20	340
cTN[i]	4.84	1.86	5.40	340
cTN[c]	7.82	3.00	6.57	345

[a] An invariant lifetime of 2.6 ns is assumed in these calculations of $k_{q(eff)}$
[b] 0.4 M NaCl, 20 mM MOPS, 2 mM EDTA (pH 7.2) ± 2 mM free Ca^{2+} ± 1 mM DTT
[c] 8 M urea, 0.3 M NaCl, 20 mM MOPS, 2 mM EDTA (pH 7.2)
[d] 0.4 M NaCl, 20 mM MOPS, 2 mM $CaCl_2$ (pH 7.2)
[e] Same solvent as in [d] but sample frozen for 1 week, thawed, then fluorescence measured
[f] 0.4 M NaCl, 20 mM MOPS, 2 mM EDTA, 2 mM DTT (pH 7.2)
[g] 0.2 M NaCl, 10 mM MOPS, 2 mM EDTA, 1 mM DTT (pH 7.2)
[h] 10 mM Pipes, 2 mM EDTA (pH 6.8)
[i] 10 mM Pipes, 2 mM $CaCl_2$ (pH 6.8)

diac system the reconstituted troponin shows quenching parameters equivalent to those of the skeletal system indicating a high degree of chromophore exposure; however, native cTN does not appear to lose as much of its structural integrity, with respect to the exposure of tryptophan residues.

The folded structure of reconstituted troponin from cardiac muscle is sensitive to Ca^{2+}. CD measurements have indicated an ~10% increase in the far UV ellipticity upon addition of this cation [5]. An analogous situation has also been demonstrated for the skeletal protein [16]. Although the far UV ellipticities of native troponin from skeletal and cardiac muscle are comparable to those of their reconstituted counterparts they differ in response to Ca^{2+}. Skeletal native troponin shows an 8–10% increase in the far UV ellipticity upon Ca^{2+} addition whereas the spectra for cardiac native troponin either in the presence or absence of Ca^{2+} agree within experimental error (unpublished).

These differences in the values for quenching constants of native reconstituted troponin are obvious reflections of subtle differences in folding and assembly of the constituent subunits which may be relevant to the biological activity of these proteins.

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