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MOTILITY OF THE N-TERMINAL TAIL OF PHOSPHORYLASE b AS REVEALED BY CROSSLINKING

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SUMMARY

There are lysyl-ε-NH_2 groups within about 3.5 Å distance across the intersubunit contact area of rabbit muscle phosphorylase b, as shown by crosslinking with malonic diimidate. These include the lysines of N-terminal region as revealed by limited tryptic digestion, but the contribution of the tail lysines to overall formation of covalent dimers is small. The fine structure of dimer band on dodecylsulfate-gelelectrophoretograms of crosslinked phosphorylases suggests that the tail retains its freedom in the phosphorylase b--AMP complex. Amidination induces the dissociation of phosphorylase b dimer, which is slow relative to crosslinking.

One of the major structural differences between phosphorylase a and b /EC. 2.4.1.1/, as revealed by X-ray crystallography, is that the N-terminal part /some twenty amino acid residues/ is fixed in the former whereas it is motile in the latter (1,2). The phosphorylation of Ser-14 by phosphorylase kinase, i.e. the b to a conversion, immobilizes the N-terminal tail and traps the enzyme in its active conformation (cf. 3,4). Alternatively, phosphorylase b can be activated by liganding with AMP (cf. 5). It is not clear whether in AMP--activated phosphorylase b the N-terminal segment retains its freedom or becomes immobilized as part of the allosteric change (2).

In a previous work on the crosslinking of phosphorylase b with bifunctional diimidates we have found that even the shortest reagent, malonic diimidate /3.7 Å/, can readily produce covalent dimers (6). From the current X-ray structure (2,7) it seems that Lys-41 in the "cap" region of one subunit and Lys-191 of the other subunit can approach each other within that distance

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/L.N. Johnson, personal communication/. A further candidate for intersubunit crosslinking is the tail region which contains two reactive amino groups in residues Lys-9 and Lys-11 (8) and extends into the symmetry related subunit in phosphorylase a (1).

In the present work we examined whether the tail was involved, and if so to what extent, in the crosslinking of phosphorylase b.

MATERIALS AND METHODS

Rabbit skeletal muscle phosphorylase b was prepared according to Fischer and Krebs (9), recrystallized three times and freed from nucleotide by Norit A treatment. The A_{260}/A_{280} ratios were in the range of 0.53-0.56 after charcoal treatment. The specific activity of the enzyme was 820 $mkat.kg^{-1}$ /i.e. 49 $U.mg^{-1}$/ as determined by the method of Illingworth and Cori (10) with 16 mM glucose-1-phosphate and 1 mM AMP. Phosphorylase a was obtained from phosphorylase b according to Krebs and Fischer (11).

Protein content was measured spectrophotometrically at 280 nm by using the absorbance coefficient 1.30 $cm^2 mg^{-1}$ (12). Malonic bis(methylimidate) hydrochloride /diimidate/ was prepared by the procedure of McElvain and Schroeder (13) from the corresponding dinitrile. Methyl acetimidate hydrochloride was the product of Pierce. Trypsin, B grade, essentially freed from chymotrypsin by treatment with diphenylcarbamoyl chloride was purchased from Calbiochem. The other chemicals were commercial preparations of reagent grade.

Crosslinking was performed at 0.2 mg/ml enzyme concentration in 0.2 M triethanolamine-HCl buffer, pH 8.0, with 10 mM malonic diimidate for 1 hour at 30°C, then the mixture was allowed to stand overnight at 0°C. Treatment with methyl acetimidate was made under the same conditions with 80 mM reagent.

Tryptic digestion of phosphorylase b or a was carried out at 0.2 mg/ml protein concentration in 0.2 M triethanolamine-HCl buffer, pH 8.0, containing 0.1 M glucose at 30°C with 5 or 10 ug/ml trypsin. At appropriate times the reaction was stopped by the addition of a fivefold molar excess of soybean trypsin inhibitor. Similar results were obtained if phenylmethylsulfonyl fluoride was used as inhibitor.

Sodium dodecylsulfate polyacrylamide gelelectrophoresis in 5.6 % gels was performed according to Fairbanks et al. (14). Staining with Coomassie brilliant blue R-250 and the densitometric evaluation of gels were made as described previously (15). Crosslinking did not alter the staining properties of phosphorylase.

RESULTS

Since the N-terminal part of both phosphorylase a and b can be split by trypsin at Arg-16 producing a truncated enzyme, phosphorylase b' (16-18), it seemed plausible that by limited tryptic hydrolysis it could be decided to what extent the tail lysines /Lys-9 and Lys-11/ were involved in the formation of covalent dimers.

Phosphorylase b was first digested with trypsin for various times /2 to 40 min/, then crosslinked with malonic diimidate. The differences in the electro-

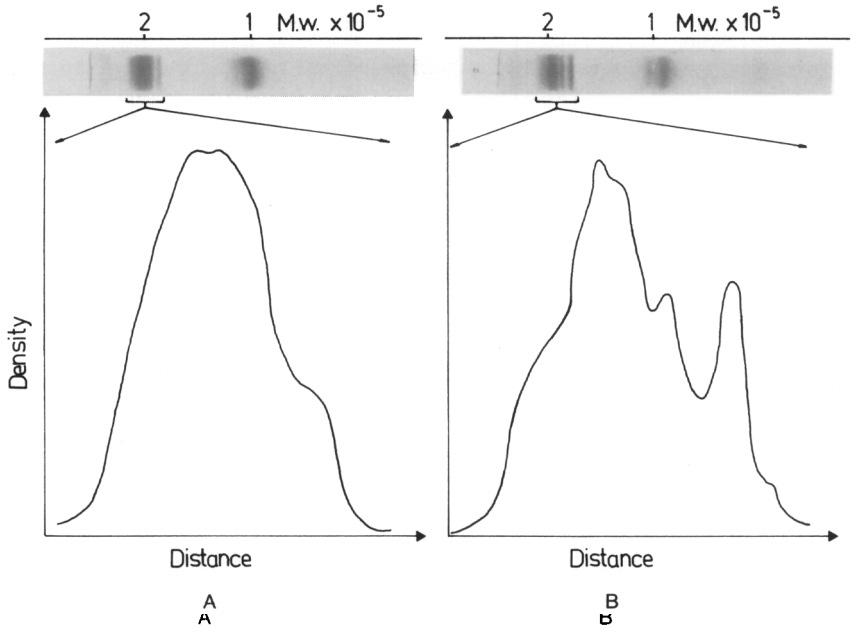

Fig.1. Crosslink patterns of phosphorylase b before and after limited tryptic digestion. Tryptic hydrolysis followed by crosslinking with malonic diimidate was performed as described in METHODS. A: no digestion; B: 5 min of tryptic (10 ug/ml) digestion. In both A and B the picture of gel and the densitogram of the dimer area (indicated at the gel by the bracket) are shown.

phoretic pattern of phosphorylase b before and after a short exposure to trypsin are seen in Fig. 1. The removal of N-terminal tail, which takes place under the given conditions (19,20), practically does not affect the amount of dimer formed but makes the dimer band more structured: the somewhat hazy picture before trypsinolysis becomes sharpened, more /4 or 5/ subbands can be discerned. This band-sharpening did not depend on the amount of protein loaded onto the gel. The subbands are due to the formation of crosslink isomers (21) whose mobilities slightly differ owing to their different shape and/or dodecylsulfate binding (22). Digestion of the enzyme with trypsin up to 40 min did not further change the crosslink pattern, there was only a moderate, even decrease in band intensities. The difference between patterns A and B in Fig.1 is then that in B certain subbands in the middle of dimer area disappear whereas the fastest subband becomes more prominent. Apparently, in phosphorylase b' some crosslink isomers, presumably those involving Lys-9 and Lys-11, cannot form whereupon the formation of another crosslink isomer corresponding to the

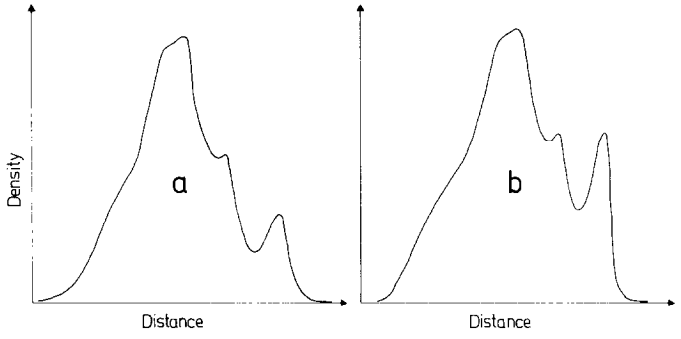

Fig.2. Densitograms of dimer areas on the gelelectrophoretic patterns of crosslinked phosphorylase a. Curve a: phosphorylase a without digestion crosslinked with malonic diimidate; curve b: phosphorylase a digested with 5 ug/ml trypsin for 10 min, then crosslinked with malonic diimidate. For details see METHODS.

fastest subband is promoted. These data suggest that the tail lysines participate in intersubunit crosslinking. The haziness of dimer area in case of the untruncated enzyme may be due to crosslinking the tail in more than one way. This would be in accord with the motile nature of the N-terminal segment. Indeed, with phosphorylase a, whose N-terminal part is fixed, the dimer area was found to be structured even before digestion and tryptic pre-treatment only brought about some intensification of the fastest subband /Fig.2/. It appears that the fine structure of dimer band reflects the motility or immobility of the N-terminal segment.

The existence of several subbands in the dimer area of phosphorylase b' does not necessarily mean that there is more than one lysyl pair involved in intersubunit crosslinking. Split bands can also be produced by intrasubunit crosslinks (23). Thus if there is one intersubunit crosslink of type α/probably Lys-41 - Lys-191, L.N. Johnson, personal communication/, and one intrasubunit crosslink of type β, then the following crosslink isomers may form: α, α_2, $\alpha\beta$, $\alpha_2\beta$, $\alpha\beta_2$ and $\alpha_2\beta_2$. Accordingly, one could account for the observed pattern by assuming one intersubunit and one intrasubunit lysyl pair whose NH_2-groups are within 3.7 Å distance. This explanation is supported by the fact that the monomer band of phosphorylase is split after treat-

ment with malonic diimidate /Fig.1/, i.e. there is at least one intrasubunit crosslink*.

Next, in the "inverse" experiment phosphorylase b was first crosslinked with malonic diimidate then exposed to limited tryptic digestion. In light of the foregoing we expected a decrease in the dimer/monomer ratio on the gel-electropherograms after proteolysis, owing to the cleavage of covalent dimers linked through the tail. Contrary to this expectation the dimer/monomer ratio increased. The explanation of this finding is that amidination dissociates the phosphorylase b dimer into monomers, provided that no intersubunit crosslink was made, and the monomers are more susceptible to proteolysis. Two lines of evidence led to this conclusion: 1/ Methyl acetimidate, a monofunctional amidinating reagent, markedly enhanced both the tryptic and chymotryptic digestibility of the enzyme. The tryptic digestion of acetimidate-treated phosphorylase is probably initiated at external arginyl bonds as amidination has been shown to protect lysyl peptide linkages (24). Incidentally, methyl acetimidate produced a faint dimer band, i.e. behaved as a crosslinker, which can only occur if there are NH_2-groups as close to each other as about 3 Å (25). 2/ Methyl acetimidate-treated phosphorylase b eluted with a trailing edge from a Sephadex G-200 column, characteristic of a dissociating system. It is important to note that dissociation of phosphorylase b must be slow relative to amidination since as much as 80 % of protein could be brought into the dimer band by malonic diimidate. The dissociation of phosphorylase b dimer upon carbamylation of its NH_2-groups has already been observed (26). As for the fine structure of dimer area in the "inverse" experiment, it became more structured as a result of tryptic digestion /Fig. 3A/, but not as pronouncedly as when proteolysis preceded crosslinking /Fig.1/.

Since the fine structure of dimer area and its change upon limited proteolysis seemed diagnostic as to the motility of the N-terminal tail, we examined the dimer band of the phosphorylase b-AMP complex crosslinked with malonic

* In 4.5 % gel the splitting of monomer band after treatment with malonic diimidate was not observed (6).

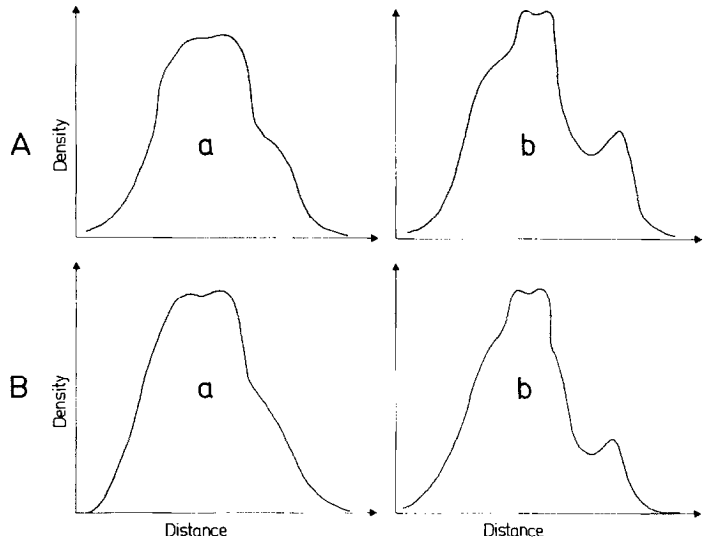

Fig.3. Densitograms of dimer areas on gelelectrophoretic patterns of crosslinked phosphorylase b (A) and phosphorylase b-AMP complex (B). Crosslinking with malonic diimidate was followed by 10 min of digestion by trypsin (5 ug/ml). Curves a: before digestion; curves b: after digestion. In B the crosslink mixture also contained 2 mM AMP. For details see METHODS.

diimidate. It is seen in Fig. 3A and B that AMP did not alter the dimer area; on trypsin treatment applied after crosslinking the patterns become more structured but have the same shape independent of whether AMP was present during crosslinking or not. It follows that in the phosphorylase b-AMP complex the tail is not immobilized giving rise to the broad, unstructured dimer area.

DISCUSSION

The data presented indicate that the tail lysines of phosphorylase b can be covalently stapled to the symmetry-related subunit. This is in keeping with the notion that this polypeptide segment conveys messages between the subunits in allosteric transitions (19). The involvement of tail-lysines in intersubunit crosslinking is relatively small, which is conceivable if the tail is motile. Namely, then the intramolecular facilitation in the reaction of the second function of the reagent bound to one of the tail lysines is relevant first of all to the other tail lysine, and the intratail staple thus produced rules out the participation of tail in intersubunit crosslinking.

The lack of band sharpening in the dimer area of the phosphorylase b-AMP complex suggests that the tail remains motile in this species. Apparently, the tail-mediated /via Ser-14/ and AMP-induced activation mechanisms of phosphorylase are different and fairly independent. Current evidence seems to support this view. Griffiths et al.(27) observed similar electron spin resonance signal when AMP bound to phosphorylase a or b. Janski and Graves (28) have found that inhibition of phosphorylase a by antibodies prepared against the N-terminal segment is markedly alleviated by AMP without dissociating the immune complex. Dreyfus et al. (29) could activate phosphorylase b' to nearly the same extent as phosphorylase b by various alcohols. It seems that the proper alignment of active site residues can be achieved by various movements in other parts of the protein molecule.

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