CROSSLINKING AND MODIFICATION OF Na,K-ATPase BY ETHYL ACETIMIDATE

Kathleen J. Sweadner †

The Biological Laboratories, Harvard University, Cambridge, Mass. 02138

Received August 22,1977

SUMMARY: Monofunctional imidoesters such as ethyl acetimidate can induce crosslinking of subunits of the (Na^++ K^+) ion-stimulated ATPase. The crosslinked product is shown to be composed of equal parts of two subunits: one phosphorylated by γ-[^{32}P]ATP, the other a glycoprotein. Because crosslinking of proteins by imidoesters normally requires reaction at both ends of a bifunctional reagent, the reaction is unexpected. A model for the reaction is proposed, in which a favorably positioned amino group on one subunit displaces the amidino group on the other, forming a covalent diamidino crosslink between the two subunits.

Reaction with imidoesters also partially inhibits the Na,K-ATPase and reduces the sensitivity of the phosphorylated form of the enzyme to potassium ion. This modification resembles the effect of ouabain, a specific inhibitor of Na,K-ATPase, and is independent of crosslinking.

INTRODUCTION: Most of the purified preparations of the ion transport Na,K-ATPase (EC 3.6.1.3) contain a protein of ~90,000 molecular weight, which can be phosphorylated by γ-[^{32}P]ATP, and a smaller glycoprotein (for review, see 1). Kyte has reported that the phosphorylatable subunit (α) can be crosslinked to the glycoprotein (β) with dimethyl suberimidate, a bifunctional imidoester (2). The experiments presented here indicate that the crosslink induced by dimethyl suberimidate in Na,K-ATPase is probably not due to the formation of a bisamidine derivative (a suberamidine bridge between the two subunits) as widely believed. That it nonetheless does crosslink two different subunits of the Na,K-ATPase is confirmed, and a model is proposed to explain the reaction. Imidate reaction was also observed to alter the phosphorylation and dephosphorylation of the enzyme.

METHODS: Canine renal medulla Na,K-ATPase, prepared by the method of Kyte (3), was the gift of S. Goldin. Canine brain microsomes were prepared by

† Present address: Dept. of Neurobiology, Harvard Medical School, Boston, Mass. 02115.

ISSN 0006-291X

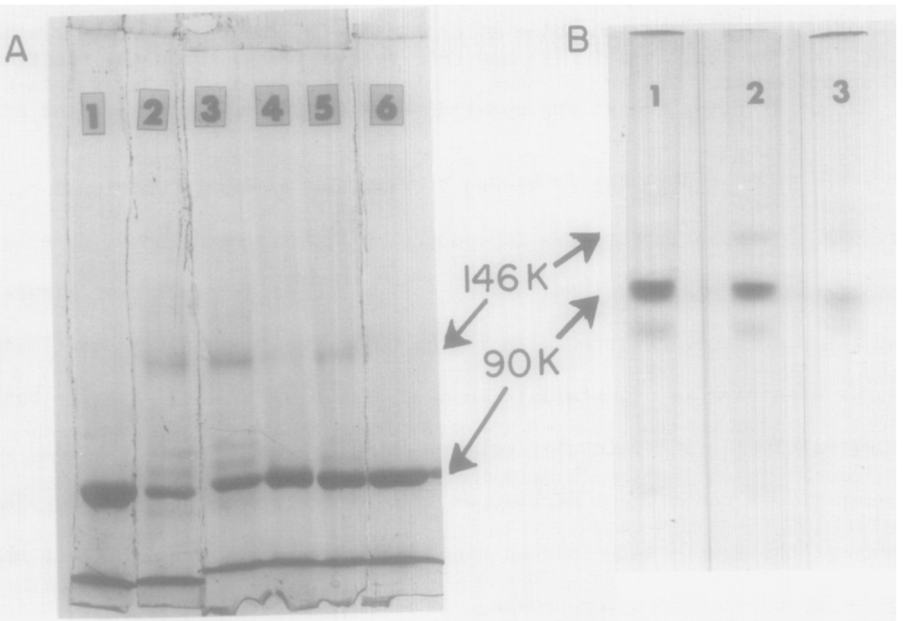


Figure 1. Crosslinking of Na,K-ATPase by Imidoesters.
1A: Purified kidney Na,K-ATPase was reacted with imidoesters for 3 hours at room temperature at the indicated concentrations in N-ethyl-morpholine acetate pH 8.5, electrophoresed in sodium dodecyl sulfate on 5% polyacrylamide, and stained with Coomassie blue. 1) Control, 2) suberimidate, 10 mM, 3) acetimidate, 10 mM, 4) mercaptobutryimidate, 2 mM, 5) dithiobispropionimidate, 5 mM, 1% β-mercaptoethanol, 6) control. Slots 1 and 2 were sliced from one slab gel, slots 3-6 were from another. The dark band at the bottom of the gel is the dye front, which includes proteins of ~70,000 molecular weight and less.
1B: Reaction was for 1½ hours at room temperature in N-ethyl morpholine acetate pH 8.5. 1) Control, 2) acetimidate, 2.5 mM, 3) 1,5-difluoro-2,4-dinitrobenzene, 0.25 mM. Electrophoresis was in tube gels of 5% polyacrylamide in sodium dodecyl sulfate.

homogenizing gray matter in 9 volumes of 0.32M sucrose 1 mM EDTA, centrifuging at 8,500 xg for 20 minutes, and centrifuging the supernatant at 30,000 rpm for 45 minutes, and collecting the pellet. Dimethyl suberimidate, methyl-4-mercaptobutyrimidate, and dimethyl-3,3'-dithiobispropionimidate were from Pierce Chemical Co.. Ethyl acetimidate and succinic anhydride were from Eastman Chemicals. 1,5-Difluoro-2,4-dinitrobenzene was from Aldrich Chemical Co.. Fluoro-2,4-dinitrobenzene, dithiothreitol, and ouabain were from Sigma Chemical Co.. Acetic anhydride was from Mallinckrodt.

Crosslinking was done essentially by the method of Kyte (2), in 0.1 or 0.2M N-ethylmorpholine acetate or triethanolamine Cl buffer, pH 8.5 or 7.5. Polyacrylamide gel electrophoresis was performed by the methods of Laemmli (pH 8.6) (4) for Figure 1a, of Ziegler et al.(pH 8.3) (5) for Figure 1b, and of Avruch and Fairbanks (pH 2.4) (6) for Figure 2 and Table 1. The phosphorylation of the Na,K-ATPase with γ-[^{32}P]ATP, and the labelling of the glyco-

protein with sodium [^{3}H]borohydride after periodate oxidation of sialic acids were performed as described in (7) and (8), respectively, after the reaction with the imidoester. The crosslinked samples were washed with 30 mM Tris-Cl pH 7.5 before starting the phosphorylation reaction, to remove traces of NH_4^+.

RESULTS: The crosslinking of kidney Na,K-ATPase by the bifunctional imidate dimethyl suberimidate was reproduced exactly as reported by Kyte (2), and in addition, a monofunctional analog of suberimidate, ethyl acetimidate, was used as a control. Figure 1a shows the unexpected result that acetimidate is just as effective as suberimidate in causing the appearance of a new band at approximately 146,000 molecular weight. Similar results were obtained with ethyl acetimidate reaction of microsomes from canine brain (not shown). The stability of the acetimidate-induced band to various treatments suggests that it may be due to a covalent crosslink. It is stable to heating at 100°C for three minutes in 5% sodium dodecyl sulfate, and it can be seen when electrophoresed in sodium dodecyl sulfate at pH 8.6 and pH 2.4. Succinylation after crosslinking does not cause it to dissociate. The band does not form if the imidate is added 15 minutes after the enzyme is dissolved in sodium dodecyl sulfate. Ruoho and Kyte have reported photochemical formation of a similar dimer (9); photochemical reaction cannot be directly responsible for the imidate crosslinking since the samples and their controls were incubated in dim light or in the dark.

Other monofunctional imidates, such as methyl-4-mercaptobutyrimidate, will cause formation of the same band (Figure 1a). Dithiobispropionimidate is a bifunctional imidate similar in length to suberimidate, but with a disulfide bond in the middle that can be broken with reducing agents. That it too causes the formation of the 146,000 molecular weight band, even in the presence of β-mercaptoethanol, is seen in Figure 1a. The same pattern was seen without β-mercaptoethanol and when β-mercaptoethanol or 100 mM dithiothreitol were added after the dithiobispropionimidate reaction (data not shown).

Other lysine-reactive reagents were used. No crosslinking was seen as a result of reaction with acetic anhydride, succinic anhydride, or fluoro-2,4-

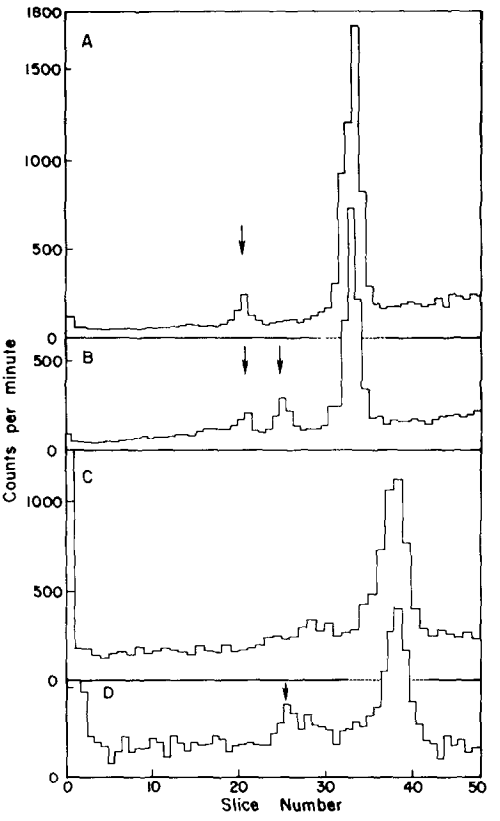


Figure 2. Identification of the Crosslinked Dimer of Kidney Na,K-ATPase. The large subunit of the Na,K-ATPase was labelled by reaction with γ-[^{32}P]ATP (A and B, main peaks). The glycoprotein subunit was labelled by periodate oxidation of sialic acids followed by [^{3}H]borohydride reduction (C and D, main peaks). Crosslinking was by 25 mM acetimidate in N-ethylmorpholine acetate pH 7.5 for 1 hour at room temperature (B and D). The peak at 146,000 molecular weight (~slice 26) is labelled by both procedures, while the peak at 180,000 molecular weight (~slice 20) is labelled only by phosphorylation (arrows). Electrophoresis was at pH 2.4 on gels of 5% polyacrylamide.

dinitrobenzene. 1,5-Difluoro-2,4-dinitrobenzene does cause the appearance of the 146,000 molecular weight band (Figure 1b). This reagent is known to be capable of forming a bridge between lysine side groups.

To find out if an $\alpha\beta$ dimer is really being formed by acetimidate reaction, the α subunit was labelled by phosphorylating it with γ-[^{32}P]ATP in the presence of sodium, and the β subunit was labelled by oxidizing its sialic acid residues

with periodate and reducing the product with sodium [^{3}H]borohydride. The amount of label in each band after sodium dodecyl sulfate gel electrophoresis was measured and compared to uncrosslinked controls. The results are shown in Figure 2. The peak at 146,000 molecular weight contains 14.9% of the total ^{32}P label and 15.8% of the total ^{3}H label, which suggests that it is composed of a 1:1 ratio of the two subunits.[1] When imidate-treated canine brain microsomes were reacted with γ-[^{32}P]ATP, potassium-sensitive phosphorylated protein was seen at molecular weights of 90,000, 138,000, and 180,000, and only at these molecular weights.

Imidates, in addition to causing crosslinking, also have an effect on the phosphorylation and dephosphorylation cycle of the Na,K-ATPase. In untreated enzyme sodium stimulates and potassium markedly depresses phosphorylation from ATP. Compared to the controls, the imidate-treated enzyme has a reduced level of total phosphorylation and a reduced sensitivity to potassium. This was seen with acetimidate as well as with suberimidate, and was the same in α and $\alpha\beta$ peaks, indicating that it may be due to reaction at a site other than the crosslinking site. The effect of the imidate reaction resembles the effect of the cardiac glycoside ouabain on phosphorylation and on the potassium sensitivity of the phosphorylated form of the enzyme (Table 1). Ouabain, however, fully inhibits the hydrolysis of ATP, while in the experiment of Table 1, imidate reaction inhibited the hydrolysis of ATP only 40-45%.

DISCUSSION: Bifunctional imidoesters have been used to determine the subunit composition of a number of oligomeric proteins (10). That the usual mechanism for formation of crosslinks requires reaction at both ends of the

[1] There is also a peak of ^{32}P label at 180,000 molecular weight. It is frequently detected in untreated material, and it is potassium-sensitive (not shown), which makes it likely that it is a form of the Na,K-ATPase and not a contaminant. Its apparent molecular weight is exactly double that of the α subunit. This, combined with the fact that it is not accompanied by the ^{3}H label of the β subunit, indicates that it is an α_2 dimer. In this experiment, the ^{32}P in the 180,000 molecular weight peak makes up 4.5% of the total Na,K-ATPase-bound ^{32}P in the control, 3.9% when the control is treated with 100 mM dithiothreitol, and 6.1% in the acetimidate-treated sample.

TABLE 1

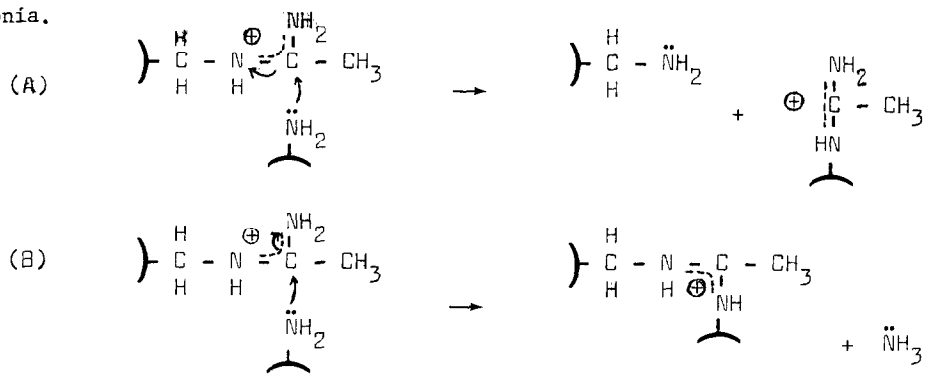
IMIDATE REACTION ALTERS SENSITIVITY TO K^+ OF THE PHOSPHORYLATED FORM OF Na,K-ATPase §

	Counts per minute in α			Counts per minute in αβ		
	(Na^+)	($Na^+ + K^+$)	Resistant to K^+	(Na^+)	($Na^+ + K^+$)	Resistant to K^+
Control	9935	1009	10.2%	-	-	-
Acetimidate	3993	1566	39.2%	359	125	34.8%
Ouabain	5531	2908	52.6%	-	-	-
Acetimidate, then ouabain	3755	2051	54.6%	390	244	62.6%
Ouabain, then acetimidate	3556	1783	50.1%	480	268	55.8%
Control	8559	1034	12.1%	-	-	-
Suberimidate	2921	1252	42.9%	632	287	45.4%

§ Acetimidate or suberimidate were reacted at 10 mM for 30 minutes at 37°C in triethanolamine Cl buffer, pH 7.5, before phosphorylation. Ouabain was 0.3 mM, and when used was also present during the phosphorylation reaction. Microsomes (brain) hydrolyze 45.6 μmoles ATP/hr/mg protein at 37°C under standard conditions (7). Before the gel electrophoresis, the potassium-sensitive incorporation of ^{32}P is 160 pmoles/mg protein; approximately 30% of the counts are recovered after slicing the gel. This data is representative of six similar experiments.

bifunctional reagent is supported by two kinds of experiment: 1) those in which the monofunctional imidoester is shown to react with the protein, but not cause any crosslinking between polypeptide chains (11-14), and 2) those in which a bifunctional imidoester containing a disulfide bond is used, and with which it can be shown that the crosslink can be dissociated by reducing agents (15-18). How, then,might ethyl acetimidate be inducing a very stable, apparently covalent crosslink between polypeptide chains? A possible mechanism is suggested by the fact that the amidino group can be displaced from proteins by ammonolysis (13, 19-21). A favorably oriented amino group on a protein can be postulated to attack the amidino group in the same way that free ammonia would, as below. Depending on which nitrogen is eliminated, the product of the reaction would be (A) transfer of the amidino group from one amino group

to the other, and (B) a covalent crosslink formed, with the release of free ammonia.



Suberimidate forms a bridge of up to 11 Å between amino groups, but the cross-linked derivative proposed here would have to be as close as 3 Å. It was shown that 1,5-difluoro-2,4-dinitrobenzene forms an αβ dimer of the Na,K-ATPase. This forms a bridge of about 5 Å (22,23), which suggests that there are nucleophilic groups in close proximity between these two subunits of the Na,K-ATPase. The fact that only this specific pattern is seen, despite the presence of numerous other membrane proteins in the brain microsome preparation, supports the argument that the imidate crosslink reflects a very tight and selective association between the two polypeptide chains.

The ouabain-like modification of the phosphorylation and dephosphorylation of the Na,K-ATPase by imidates may prove useful in the elucidation of the enzyme's molecular mechanism.

ACKNOWLEDGMENTS: I would like to thank Prof. Guido Guidotti for his support, advice, and encouragement. This work was supported by grants from the National Institutes of Health (HL 08893) and from the National Science Foundation (BMS 06752) to Guido Guidotti. KJS was supported by United States Public Health Service Training Grant GM 138.

REFERENCES:

1. Jorgensen, P.L. (1975) Q. Rev. Biophys. 7, 239-274.
2. Kyte, J. (1972) J. Biol. Chem. 247, 7642-7649.
3. Kyte, J. (1971) J. Biol. Chem. 246, 4157-4165.
4. Laemmli, U.K. (1970) Nature 227, 680-685.
5. Ziegler, A., Harrison, S.C., and Leberman, R. (1974) Virology 59, 509-515.
6. Avruch, J., and Fairbanks, G. (1972) Proc. Nat. Acad. Sci. 69, 1216-1220.
7. Jorgensen, P.L. (1974) Biochim. Biophys. Acta 356, 53-67.

8. Steck, T.L., and Dawson, G. (1974) J. Biol. Chem. 249, 2135-2142.
9. Ruoho, A., and Kyte, J. (1974) Proc. Nat. Acad. Sci. 71, 2352-2356.
10. Davies, G.E., and Stark, G.R. (1970) Proc. Nat. Acad. Sci. 66, 651-656.
11. Dutton, A., Adams, M., and Singer, S.J. (1966) Biochem. Biophys. Res. Comm. 23, 730-739.
12. Hartman, F.C., and Wold, F. (1967) Biochemistry 6, 2439-2448.
13. Ji, T.H. (1974) J. Biol. Chem. 249, 7841-7847.
14. van Driel, R., and van Bruggen, E.F.J. (1975) Biochemistry 14, 730-735.
15. Wang, K., and Richards, F.M. (1974) J. Biol. Chem. 249, 8005-8018.
16. Wang, K., and Richards, F.M. (1975) J. Biol. Chem. 250, 6622-6626.
17. Bragg, P.D., and Hou, C. (1975) Arch. Bioch. Biophys. 167, 311-321.
18. Ruoho, A., Bartlett, P.A., Dutton, A., and Singer, S.J. (1975) Biochem. Biophys. Res. Comm. 63, 417-423.
19. Ludwig, M.L., and Byrne, R. (1962) J. Amer. Chem. Soc. 84, 4160-4162.
20. Bickle, T.A., Hershey, J.W.B., and Traut, R.R. (1972) Proc. Nat. Acad. Sci. 69, 1327-1331.
21. Reynolds, J.H. (1968) Biochemistry 7, 3131-3135.
22. Berg, H.C., Diamond, J.M., and Marfey, P.S. (1965) Science 150,64-67.
23. Wold, F. (1972) Methods in Enzymology 25, 623-651.